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(Summary Report and Prospectus)
On the basis of work preceding the present project, the hypothesis was proposed that predicts the possibility of developing a specific biochemical methodology capable of detecting early and specific signals in chromatin proteins that precede cellular toxicity or malignant transformation, caused by toxic agents or by other environmental factors (e.g., radiation). This long-range aim has been approached during the presently completed grant period by the identification of covalent modification of chromatin proteins by a specific enzymatic process of ADP-ribosylations and poly (ADP)-ribosylation to be the most probable biochemical area that can signal toxic effects leading to either cellular damage or malignant transformation. The experimental basis of this continuously tested working hypothesis is: a.) the

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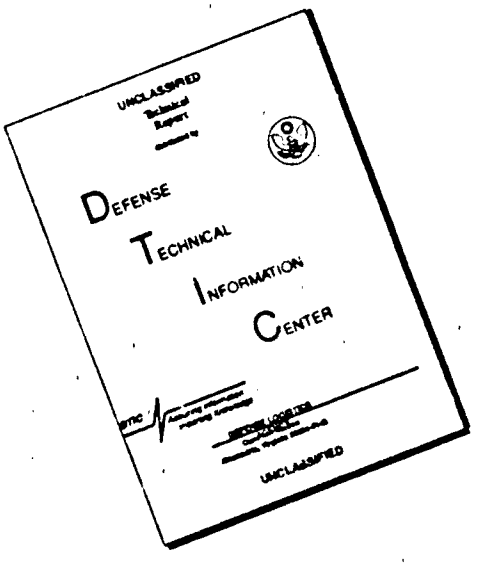

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Immunochemical and more recently chemical localization and determination of chromosomal non-histone proteins as specific nuclear acceptors of ADP-ribose and of its polymers; b.) by the detection of specific perturbations in ADP-ribosylations of non-histone proteins during early carcinogenesis, development and hormonal influences, biological areas that are recognized to express differentiated cellular processes. Since the signals obtained during early carcinogenesis, development and hormone actions are clearly distinguishable, there is a predictably high probability that highly specific macromolecular mechanisms will become identificable during subsequent research, that is aimed to discover a new experimental basis for the discrimination between nuclear processes connected to physiological function in distinction to responses to toxic and carcinogenic injuries. The specific report is presently concerned with the following results: 1. Immunochemical demonstration of the sub-nuclear distribution of $(ADP\text{-}R)_n$, identifying non-histone proteins as specific acceptors. 2. Direct chemical methodology that identified actin as one of many non-histone proteins as an ADP-R acceptor. 3. Chemical demonstration of increased protein-ADP-ribosylation during early precancerous state. 4. Enzymological demonstration of age-dependent differences in poly(ADP-R) biosynthesis in isolated cardiocyte nuclei. 5. Hormonal influences of nuclear ADP-ribosyations, identifying organ specific (cardiocyte nuclei-specific) inhibitory effect of aldosterone on ADP-ribosylation. 6. Inhibitory effects of hypophysectomy and adrenolectomy on nuclear poly ADP-ribosylation.

→ Keywords: Actin, Biosynthesis, Hormones, Aldosterone, Enzymes, Inhibitors, Hypophysectomy, Adrenolectomy (AW)

B

DEVELOPMENT OF AN ASSAY FOR THE EARLY DETECTION OR ORGAN SPECIFIC CARCINOGENESIS BY THE DETERMINATION AND TURNOVER OF NAD^+ AND POLYADENOSINE DIPHOSPHORIBOSE

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PROGRESS REPORT

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The *in Vivo* Distribution of Immunoreactive Larger Than Tetrameric Polyadenosine Diphosphoribose in Histone and Non-histone Protein Fractions of Rat Liver*

(Received for publication, February 23, 1979)

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The macromolecular association of immunoreactive naturally occurring polyadenosine diphosphoribose $n > 4$ with histones and non-histone proteins was determined with the aid of an improved method of extraction of polyadenosine diphosphoribose and a combination of radioimmunoassay and molecular filtration. More than 99% of the naturally occurring polyadenosine diphosphoribose $n > 4$ was present in rat liver in covalent association with non-histone proteins. The chain length of the polymer varied between $n = 4$ and $n = 34$. Less than 1% of naturally occurring polyadenosine diphosphoribose $n > 4$ was almost evenly distributed between histone fractions f1, f2a, f2b, and f3. Adenosine diphosphoribose polymers of relatively long chain length were also detected in the histone fractions. The covalent association of polyadenosine diphosphoribose with non-histone proteins was demonstrated by affinity chromatography.

Polyadenosine diphosphoribose, the homopolymer of ADP-R,[1] is a unique natural product of eukaryotic cells that is formed from NAD^+ and is localized in the nucleus (1, 2). Mitochondria contain a trace amount of an ADP-R-protein adduct that is also derived enzymatically from NAD^+ (3). Extensive evidence obtained with the aid of a variety of chromatographic and electrophoretic techniques applied to several types of cultured cells and to animal tissues supports the view that polyadenosine diphosphoribose exists in chromatin as a covalent protein adduct (4–13). This conclusion is substantiated by the fact that the enzymatic synthesis of the polymer occurs on a protein primer (1, 2, 14). Although the precise nature of the bond or bonds between the polymer and proteins remains unknown, it is generally recognized that the protein-polyadenosine diphosphoribose adducts are unstable at alkaline pH (1, 2). The detection of fragments of free polyadenosine diphosphoribose synthesized *in vitro* by isolated nuclei of mouse liver does not necessarily reflect conditions which exist *in vivo* and could be an artifact of isolation (15).

One of the fundamental unresolved problems is the identification of nuclear proteins which are susceptible to polyadenosine diphosphoribosylation. Histones (4–8) and non-histone proteins (10, 12, 13) have been reported to contain covalently bound oligo- and polyadenosine diphosphoribose as detected by radioactive labeling techniques. Identification of a histone f1 dimer joined by a 16 ADP-R-containing oligomer (16, 17) appeared to confirm the acceptor role of basic nuclear proteins. On the other hand the ADP-R histone f1 adduct identified previously as a Schiff base (18) was shown to play the role of an elongation primer of the highly purified polyadenosine diphosphoribose polymerase (14), implying a possibly different biochemical function for mono- and oligomeric ADP-R-histone adducts. Highly purified enzymes (14, 19–21) synthesize *in vitro* only very short oligomers, containing an average of 1 to 3.7 ADP-R units except in the presence of histone f1-ADP-R Schiff base (18), whereupon oligomers of an average of 10 to 12 ADP-R units may be formed (14). Isolated nuclei of pigeon liver,[2] however, readily synthesize polymers of an average chain length of 40 ADP-R units (22, 24) and various preparations of nuclei from rat liver and calf thymus under certain conditions can produce polymers of an average chain length of 65 ADP-R units (25). Extrapolation of these results to *in vivo* conditions tend to suggest that more complex polymers of ADP-R may be formed in animal tissues than predicted from presently known *in vitro* enzymology. This assumption is supported by the discovery of branched or cross-linked polyadenosine diphosphoribose (26).

We have chosen to determine the polyadenosine diphosphoribose content of dilute acid-soluble and -insoluble proteins of rat liver because it would be anticipated that the macromolecular distribution of the polymer under *in vivo* circumstances would reflect a physiological condition. In contrast to the majority of attempts to quantitate polyadenosine diphosphoribose by radiochemical analyses only, we have recently adopted a highly specific anti-polyadenosine diphos-

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[1] The abbreviations used are: ADP-R, adenosine diphosphoribose; IgG, immunoglobulin G.

[2] The advantage in terms of specificity of the IgG globulin (22) over other antisera (9, 23) is probably traceable to the unusual nature of pigeon liver nuclei used for the synthesis of the antigen. Pigeon liver nuclei, as distinguished from rat liver nuclei, can deaminate nicotinamide to nicotinic acid, thus, the expected product inhibition of the polymerase reaction by nicotinamide is minimized, resulting in the synthesis of apparently more specifically antigenic polyadenosine diphosphoribose.

phoribose IgG (22) for the quantitative analysis of the polymer.[1]

Radioimmunoassays for polyadenosine diphosphoribose have been described by others (9, 23); however, the antigens used were much shorter oligomers than employed in our work (22), and antisera were significantly less specific than the IgG (22). This difference in specificity between previously developed antisera (23) and the IgG (22) is illustrated by the fact that displacement binding curves by ADP-R oligomers varying in average chain lengths between 6 and 28 ADP-R units were dissimilar (23), whereas no such variation was detectable with the IgG (22). With our radioimmunoassay, the quantity of larger than tetrameric polyadenosine diphosphoribose can be determined with the same sensitivity.

The present report is concerned with the determination of polyadenosine diphosphoribose in rat liver protein fractions separable by their solubility in dilute acids. Special efforts were made to minimize chemical or enzymatic (1, 2, 27) reactions which would result in a loss or partial degradation of the protein-bound polymer during its isolation.

MATERIALS AND METHODS

Male Sprague Dawley rats of 200 g body weight were deprived of food for 24 h prior to the removal of livers for analyses. After pentobarbitol anesthesia livers were immediately freeze-clamped *in situ* with aluminum tongues kept at liquid N_2 temperature. The quick-frozen liver samples were either freeze-dried immediately or instantly fractionated as follows.

I. Isolation of Acceptor-free Immunoreactive Polyadenosine Diphosphoribose from Total Rat Liver, Acid Extracts and Sediments

Freeze-dried liver powder from *in situ* freeze-clamped organs was prepared as described (22). Two grams of anhydrous powder were homogenized in either 25 ml of 5% $HClO_4$ or 50 ml of 0.25 N HCl for 3 min in a container immersed in an ice bath, with Polytron homogenizer (Brinkmann) at half-maximal speed. The homogenates were stirred mechanically for 30 min at 0°C (ice bath) and the insoluble sediment was separated by centrifugation. Extracts prepared by 5% $HClO_4$ were centrifuged at 1,100 × *g* for 30 min at 4°C, whereas 0.25 N HCl extracts were sedimented at 12,000 × *g* for 30 min at 4°C. The sediments were re-extracted twice exactly as described for the first extraction. The combined extracts were concentrated by ultrafiltration (Amicon Diaflo, UM-2 membrane) to less than 10 ml volume in an ice bath, dialyzed twice at 4°C against 4 liters of H_2O for 10 h, then freeze-dried. The freeze-dried acid extracts, the sediments, or 0.2 g of unfractionated total dry powder were digested as follows. The samples were suspended in 3 ml of N NaOH and digested for 24 h at 37°C. The pH was adjusted to 8.5 with 3 N HCl and Tris base (approximately 300 mM, final concentration) and $MgCl_2$ was added to a final concentration of 10 mM. Combined digestion with DNase (90 μg/ml, EC 3.1.4.5, Sigma), micrococcal nuclease (12 μg/ml; EC 3.1.4.7, Sigma), and alkaline phosphatase (30 μg/ml, chromatographically purified EC 3.1.3.1, Worthington Biochemicals) was carried out by incubation for 6 h at 37°C. At this time the incubation mixtures were recharged with exactly the same quantities of nucleases and the incubation-digestion continued overnight at 37°C. EDTA (20 mM, final concentration), sodium dodecyl sulfate (0.25%, final concentration) and proteinase K (Merck Co., Germany, supplied by Beckman Co.; 1.7 mg/ml, final concentration) were then added and digestion continued for 12 h at 37°C with vigorous shaking. At this step the acid-extractable samples were completely digested and ready for phenol extraction (22). The insoluble material present in the digests of total liver powders was sedimented by centrifugation, the supernatant was saved, and the sediment after re-suspension in 5 ml of 500 mM Tris-HCl (pH 8.5) was redigested with proteinase K for 12 h at 37°C. This procedure was repeated two to five times, resulting in completely soluble digests, which were combined. All digests were extracted with water-saturated phenol (1 ml of phenol/4 ml of digest), layers were separated by centrifugation (3,000 rpm for 10 min), and the phenol phase was back-extracted with water (1 ml of H_2O/2 ml of phenol extract). The combined aqueous fractions were re-extracted with water-saturated phenol the second time, and back-extracted with water as before. The combined aqueous extracts were freed from phenol with water-saturated diethyl ether (3 ml of ether/1 ml of original digest) and the ether phase was back-extracted with water (1 ml of H_2O/10 ml of ether fraction). The combined aqueous extracts were extensively dialyzed (at 4°C for 24 h) against five changes of 100-fold excess H_2O and then freeze-dried. The resulting dried sediment containing the extracted polyadenosine diphosphoribose was dissolved in 1 ml of 1 mM phosphate buffer (pH 7.0) and the radioimmunoassay was performed by the nitrocellulose membrane filtration technique as described (22). Storage of whole frozen liver samples at −15°C for several days or isolation of nuclei by the nonaqueous technique described earlier (22) resulted in an uncontrollable variation of the immunoreactive polyadenosine diphosphoribose content, indicating that degradation of macromolecular polyadenosine diphosphoribose in water containing tissue samples can take place either during storage or during the elaborate process of the application of the colloid mill and subsequent density gradients in organic solvents (22). For this reason the more rapid and conservative extraction procedure used in the present study resulted in a smaller deviation in analytical results as compared to our previous report (22). The recovery of polyadenosine diphosphoribose $n = 40$ added to acidic extracts, prepared by the methods described below was between 85 and 95%, compared to a recovery of 83 to 123% obtained in the preceding report (22). This indicated that the apparent variance between present and earlier results was due to the previously uncontrollable decay of the polymer within tissue samples rather than inconsistencies in the radioimmunoassay itself.

The method of fractionation of acid-extractable proteins by carboxymethylcellulose chromatography and by organic solvents was adopted from standard procedures (28–30).

II. Quantitative Determination of Polyadenosine Diphosphoribose $n > 4$ in Histone Fractions

A. Carboxymethylcellulose Column Chromatography—Two grams of dry powder of rat liver were extracted by homogenization in the Polytron instrument three times with 50 ml of 0.25 N HCl and the combined extracts were concentrated by ultrafiltration as described under I above. The concentrated extract, about 7.5 ml final volume, was dialyzed overnight against 4 liters of 0.1 M sodium acetate buffer (pH 4.2) at 4°C and applied to a carboxymethylcellulose column (2 × 45 cm) which had been equilibrated with the same buffer. Non-histone proteins were quantitatively removed by the equilibration buffer as followed by the Brinkmann UV monitor 605 until all UV-absorbing material (at 280 nm) was eluted (28). Histones f1, f2, and f3 were eluted stepwise with 0.17 M sodium acetate buffer (pH 4.2) containing 0.42 M NaCl, 0.01 N HCl, and 0.02 N HCl, respectively, at a flow rate of about 30 ml/h (8-ml fraction/tube). Each fraction containing non-histone proteins and histones f1, f2, and f3 were dialyzed overnight against distilled water and then freeze-dried. The freeze-dried samples were digested and assayed as described under I.

B. Separation of Histone-containing Fractions by Organic Solvents—Two grams of anhydrous liver powder were extracted three times with 5% $HClO_4$ and the combined extracts, after concentration by ultrafiltration (see I), were treated with trichloroacetic acid (final concentration 20%). The histone f1 fraction was precipitated by centrifugation at 10,000 × *g* for 30 min at 4°C. The sediment remaining after the extraction of histone f1 was resuspended in 30 ml of ethanol, homogenized for 3 min, kept overnight at 4°C, and then sedimented by centrifugation at 1,100 × *g* at 4°C. After two additional extractions with 20 ml of 6[illegible]thanol, the combined supernatants were concentrated by vacuum [illegible]poration to about 10 ml and precipitated by 5 volumes of cold (0°C) acetone and 0.35 ml of concentrated HCl. The precipitate, after separation at 10,000 × *g* for 30 min at 4°C, was washed once in acetone and redissolved in 7 ml water. Ethanol and hydrochloric acid were added to give a final concentration of 80% and 0.25 N, respectively. The solution was again dialyzed overnight against 200 ml of ethanol at 4°C. After repeated dialysis against 200 ml of ethanol for 4 h, the histone f3 fraction was separated by centrifugation

[1] Pulse labeling of rats *in vivo* with [^{14}C]ribose indicates that NAD$^+$ and the polymer are labeled at approximately the same rates (apparent $t_{1/2}$ of ribose incorporation into NAD$^+$ and the polymer is 2.7 and 3.1 h, respectively). Differences in rates of labeling of polymers of larger and smaller molecular weights are also apparent, suggesting differences in metabolic turnover of polymers with different molecular weights. These results cast doubt on the adequacy of radiolabeling alone as a measure of the relative quantities of polyadenosine diphosphoribose in subnuclear protein fractions (unpublished results).

at 1,100 × *g* at 4°C, the supernatant was concentrated *in vacuo* to about 10 ml, and histone f2a was precipitated by 4 volumes of cold acetone. Histone f2a was collected by centrifugation at 10,000 × *g*. The remaining sediment following ethanol extraction of histones f2a and f3 contained histone f2b and was extracted by homogenization for 3 min in 10 ml of 0.25 N HCl at 4°C. The homogenate was centrifuged at 1,100 × *g* and the sediment was re-extracted three times in the same manner. Histone f2b was precipitated by addition of 5 volumes of cold (4°C) acetone and collected by centrifugation at 10,000 × *g* at 4°C. All separated histone fractions were digested and assayed as described under I.

C. Determination of Polyadenosine Diphosphoribose Content of Purified Histone f1—A 5% $HClO_4$ extract from 18 g of anhydrous rat liver powder was prepared and concentrated to approximately 10 ml as described under I, then precipitated with trichloroacetic acid as described under IIB. Pure histone f1 and contaminant non-histone peptides were sedimented by centrifugation at 10,000 × *g*. The supernatant was dialyzed overnight extensively against water and freeze-dried. The precipitate was dissolved in 2 ml of 0.1 M sodium acetate buffer of pH 4.2 and then applied to a carboxymethylcellulose column (2 × 45 cm). Non-histone proteins were separated by the buffer and histone f1 was eluted with 0.17 M sodium acetate buffer containing 0.42 M NaCl. Non-histone and histone fractions were then freeze-dried, digested, and assayed as described under I.

D. Chain Length Estimation of Naturally Occurring Polyadenosine Diphosphoribose—Three batches of *in vitro*-synthesized polyadenosine diphosphoribose of chain lengths averaging 34, 16, and 12 ADP-R units were prepared (22). The elution pattern of the three samples of polyadenosine diphosphoribose were determined on a Sephadex G-50 column (1 × 100 cm), which served as calibration (Fig. 2*A*). Approximately 1 μg of acceptor-free endogenous polyadenosine diphosphoribose was isolated from the sediment remaining after extraction with 0.25 N HCl. The polyadenosine diphosphoribose isolated from the non-histone sediment was dissolved in 2 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 1 M NaCl and applied to a Sephadex G-50 column (1 × 100 cm) which had been equilibrated with the same buffer and eluted at a flow rate of 12 ml/h (Fig. 2*B*). A 5-ml aliquot/fraction was collected. The contents of each tube from Tubes 7 through 23 were dialyzed overnight against H_2O, freeze-dried, digested, and assayed as described under I. Purified histone f1 fraction was obtained from 30 g of liver powder as described under IIC. Approximately 300 ng of polyadenosine diphosphoribose present in association with histone f1 was applied on a Sephadex G-50 column and eluted in the same way as described above. The contents of three successive tubes were combined (Tubes 8 through 12, Tubes 13 through 17, and Tubes 18 through 22), dialyzed, freeze-dried, digested, and assayed as described under I. Results are shown in Figs. 2*C*. Techniques related to the isolation, molecular filtration, determination of average chain lengths of polymers, and immunological properties were the same as reported in the preceding paper (22).

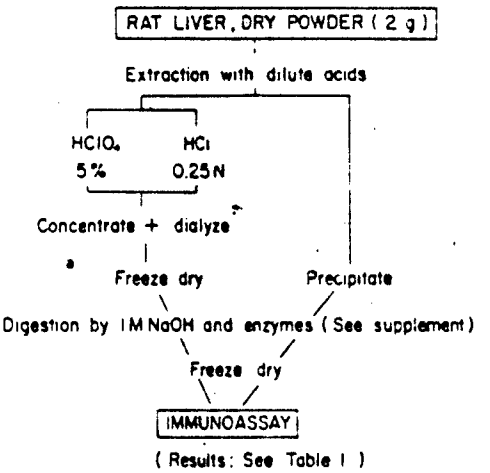
Dihydroxyboryl affinity chromatography of protein-bound polyadenosine diphosphoribose was adopted from Havaishi (31) using a phenylboronate derivative of Bio-Gel P-6 purchased from Bio-Rad.

III. Chromatography of Acid-insoluble Proteins on Dihydroxyboronate Affinity Resin—One gram of freeze-clamped liver was homogenized in 20 ml of ice-cold 5% $HClO_4$ with a Polytron (Brinkmann) homogenizer at half-maximal speed for 3 min. The resulting homogenate was centrifuged at 1,100 × *g* for 30 min at 4°C. The supernatant was discarded and the precipitate was washed three times with 20 ml of ice-cold 5% $HClO_4$ to remove soluble nucleotides. The precipitate, 0.6 g, was dissolved in 5 ml of 6 N guanidine hydrochloride containing 100 mM Tris base and the pH was adjusted to 8.2 with 6 N KOH, during continuous mechanical agitation. The suspension then was further stirred for 15 min at room temperature and centrifuged at 2,000 × *g* at 4°C for 10 min to remove $KClO_4$. The resulting clear supernatant was applied to a column (25 × 1 cm) of Affigel 601 (Bio-Rad) equilibrated with 6 N guanidine hydrochloride, 100 mM Tris, pH 8.2, at room temperature. Ambient conditions were used since previous experiments had shown better retention of polyadenosine diphosphoribose $n = 34$ on the column at 25°C than at 0–4°C. The column was washed with 100 ml of equilibrating buffer at a flow rate of 50 ml/h. This removed RNA, DNA and the bulk of protein containing no ADP-R protein adducts. The buffer then was changed to 6 N guanidine hydrochloride, 100 mM sodium phosphate, pH 5.9, and the column was eluted with 125 ml of this solution. This second eluate was concentrated and diafiltered with distilled water in an Amicon ultrafiltration cell using a UM-2 filter at 4°C overnight. The resulting solution then was freeze-dried and resuspended in 1 ml of distilled water and dissolved by adding NaOH to a final concentration of 1 N. This solution was stirred for 3 h at room temperature, brought to pH 8.4 with 1 N HCl and 100 mM Tris buffer, and then treated as under I. Polyadenosine diphosphoribose was determined by the immunoassay (22).

RESULTS AND DISCUSSION

The polyadenosine diphosphoribose content of animal tissues is subject to considerable variation, depending on the animal species assayed, and it is also recognized that the polymer undergoes fairly rapid metabolism.[1] Even within one species, analysis of the immunoreactive larger than tetrameric polymer in the same organ can yield variable results unless the rapid extraction technique is followed (see "Materials and Methods"). Conditions used by others for the determination of neutral (pH 7.5) hydroxylamine sensitivity of histone f1 and non-histone protein-ADP-R bonds (32, 33) do not assure the preservation of larger than tetrameric polymers because inactivation of the polymer degrading enzymes is uncertain (22). For this reason we avoided these techniques in our extraction of the immunoreactive polymer from acid-soluble and -insoluble proteins. Following the procedure described under "Materials and Methods" the total immunoreactive larger than tetrameric polymer content of rat liver was found to be 9,121 (S.D. ± 850) ng/g dry liver powder or 556 (S.D. ± 52) ng/mg DNA, or about 1,500 ng/g wet liver weight, based on analyses of 20 rats.

The separation of naturally occurring protein-associated polyadenosine diphosphoribose into acid (5% $HClO_4$ or 0.25 N HCl)-soluble and -insoluble fractions is illustrated in Flow Sheet 1. As shown in Table I, the 5% $HClO_4$- or 0.25 N HCl-soluble protein-associated polymer comprises only 0.9 or 1.9%.



FLOW SHEET 1. Fractionation into acid-soluble and -insoluble components.

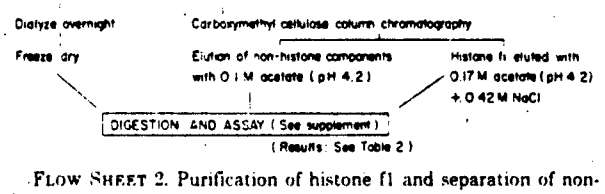
TABLE I

Polyadenosine diphosphoribose in acid extracts and sediments of rat liver powder

Separation of protein bound polyadenosine diphosphoribose of rat liver into acid-soluble and -insoluble (sediment) fractions by methods described under "Materials and Methods." Each value is the average of two analyses.

Extracting acid	Polyadenosine diphosphoribose $n > 4$		
	Supernatant	Sediment	Total
	ng/g dry powder		
5% $HClO_4$	90.0	9,600	9,700
0.25 N HCl	166.0	8,600	8,700

respectively, of the total polyadenosine diphosphoribose content of the liver. The predominant amount remained in the acid-precipitable tissue fraction. Since analyses of acid-precipitable and -soluble fractions from liver powder were performed on the same samples and recoveries were nearly quantitative, as shown by recovery of added exogenous polyadenosine diphosphoribose, it was apparent that treatment with dilute acids at 0–5°C did not decompose polyadenosine diphosphoribose. This finding is consistent with published results (1). It was expected that 0.25 N HCl more extensively than 5% $HClO_4$ would extract proteins other than histones derived from nuclear membranes, mitochondria, and microsomes (34). We found that the radioimmunoassay (22) did not indicate the presence of polymeric adenosine diphosphoribose in extranuclear cell fractions, including mitochondria which contain a specific protein associated monomer (3). It, therefore, was concluded that all immunoreactive macromolecular protein-associated polyadenosine diphosphoribose originated from nuclei, confirming earlier reports which confined the localization of the polymer to nuclei (1, 2). Extracts prepared with 5% $HClO_4$ are known to contain histone f1 (28), nuclear peptides of relatively small molecular weight (3000) which are not precipitated by 20% trichloracetic acid (35) and non-histone proteins exhibiting high electrophoretic mobility (36, 37). The procedure followed to separate these components of extracts obtained by dilute acids is illustrated in Flow Sheet 2. The chromatographic separation of histone f1 on carboxymethylcellulose (28) was performed at pH 4.2 as a precaution to prevent the cleavage of alkali unstable bonds between proteins and polyadenosine diphosphoribose (37). As shown in Table IIA, a relatively significant portion of the acid-soluble immunoreactive polymer was found in the tissue extract prepared with 5% $HClO_4$ which was not precipitable by 20% trichloracetic acid, even with bovine serum albumin as a co-precipitant, suggesting the presence of polyadenosine diphosphoribosylated peptide fragments. About one-third of the immunoreactive polyadenosine diphosphoribose associated with the 20% trichloracetic acid-precipitable fraction of the $HClO_4$ extract was identified as non-histone protein-bound polyadenosine diphosphoribose adduct by carboxymethylcellulose chromatography. Covalent association of larger than tetrameric polyadenosine diphosphoribose with histone f1 was ascertained in both $HClO_4$ and HCl extracts. Dilute HCl extracted significantly larger quantities of macromolecularly associated immunoreactive polyadenosine diphosphoribose, which was mainly recovered in the soluble non-histone fraction (Table IIB), probably representing HCl-soluble proteins of nuclear membranes. Ethanol and acetone fractionation (29, 30) of extracts prepared from precipitates after separation of histone f1 by 5% $HClO_4$ (see "Materials and Methods") yielded



FLOW SHEET 2. Purification of histone f1 and separation of non-histone components.

TABLE II

Polyadenosine diphosphoribose content of acid-soluble tissue fractions of rat liver powder

Further fractionation of acid-soluble protein-bound polyadenosine diphosphoribose present in 5% $HClO_4$ (A) and in 0.25 N HCl (B) extracts. In A the extract was divided into trichloracetic acid (20%)-soluble (supernatant) and -insoluble (precipitate) fractions. The precipitate was resolved into large non-histone peptides and histone f1 by chromatography on carboxymethylcellulose (see "Materials and Methods"). The discrepancy (13 ng/g) between total polyadenosine diphosphoribose in A and the sum of non-histone-bound and histone f1-bound polymer is due to incomplete recovery (85%) by the combined operations. The same error in combined techniques explains in B the apparent discrepancy between total bound polyadenosine diphosphoribose content and the sum of non-histone polypeptides and histone-associated polymer (24.6 ng/g). Each value is the average of two analyses.

Method of extraction and fractionation	Polyadenosine diphosphoribose $n \geq 4$
	ng/g dry powder
A. 5% $HClO_4$ extract	90.0
20% Trichloracetic acid supernatant of $HClO_4$ extract	60.6
20% Trichloracetic acid precipitate of $HClO_4$ extract	
Non-histone peptides	5.2
Purified histone f1	11.8
B. 0.25 N HCl extract	166.0
Non-histone fraction	96.3
Histone f1	16.0
Histone f2 + f3 fraction	29.5

polyadenosine diphosphoribose-containing histone fractions in amounts of 21.7 (f1), 12.2 (f2a), 22.2 (f2b), and 18.7 (f3) ng of immunoreactive polymer/g of dry liver powder. The chromatographic technique (28) and fractionation with organic solvents (29, 30) for histones yielded 45.5 and 74.5 ng of total histone-bound polyadenosine diphosphoribose/1 g of liver powder, respectively. This discrepancy corresponds to the lower limit (65%) of recovery of added polymer and is due to the technical problems of sample collection.

Bredehorst *et al.* (33) reported that, according to their technique, the predominant acceptors of adenosine diphosphoribose were found to be non-histone nuclear proteins and histones contained only small amounts of covalently bound adenosine diphosphoribose. These results are in agreement with our analyses with respect to the nature of acceptors of adenosine diphosphoribose. In contrast to their report (33), however, we consistently find polyadenosine diphosphoribose $n > 4$, not the monomer, in association with non-histone proteins. This apparent discrepancy is probably due to differences in the treatment of tissue samples, requiring utmost care in avoiding the degradation of the polymer (compare "Materials and Methods" with Ref. 33). This point is illustrated also by comparison of previous analytical results (22) with those described in the present paper which show a 10-fold higher polymer content when the conservative extraction technique (see "Materials and Methods") is strictly followed.

In vitro studies by others (15) have also shown that the specific activity of polyadenosine diphosphoribose incorporated into non-histone proteins was higher than that of histone proteins. This observation was also correlated with a longer mean polymer chain length in non-histone proteins (15).

Since the predominant portion of immunoreactive larger than tetrameric polyadenosine diphosphoribose was present in the dilute acid-precipitable non-histone protein fraction containing also nucleic acids, it was of importance to ascertain that the radioimmunoassay was not falsified by interfering nucleic acid fragments. Sample digestion in 1 M NaOH eliminates RNA and the remaining UV-absorbing material is

comprised of DNA fragments exhibiting a gel filtration pattern as shown in Fig. 1*A*. Digestion with one cycle of nucleases and dialysis (see "Materials and Methods") almost completely eliminates macromolecular UV-absorbing material (Fig. 1*B*), yet the radioimmunoassay shows the existence of polydisperse macromolecular ADP-R polymers (Fig. 1*C*). The amounts of polyadenosine diphosphoribose determined by the radioimmunoassay is so small that it cannot be detected by absorbance measurements at 260 nm. In routine work the redigestion cycle with nucleases was repeated at least twice without measurable change in immunochemically detectable polyadenosine diphosphoribose values (compare with Fig. 2*B*); thus, interference by DNA fragments can be excluded. It should be noted that DNA significantly interferes with the radioimmunoassay only when present in excess of 13,500-fold over polyadenosine diphosphoribose (22).

The macromolecular distribution of immunoreactive polyadenosine diphosphoribose isolated from acid-insoluble non-histone proteins (*B*) and histone f1 (*C*) as determined by molecular filtration on Sephadex G-50 (22) and compared to standards (*A*) is illustrated in Fig. 2. Both fractions exhibited polydispersity between average chain lengths of $n = 4$ to $n = 34$.

On the basis of a large body of evidence (4–8, 10–13, 17–25, 31, 32), it can be assumed that the polymers associated with acid-soluble and -insoluble proteins represent polyadenosine diphosphoribose predominantly covalently bound to proteins.

We have attempted to confirm the covalent association of polyadenosine diphosphoribose with non-histone proteins by an alternative method employing fractionation of acid-insoluble rat liver proteins on a dihydroxyboryl affinity column under denaturing conditions (31). Using the procedure outlined under "Materials and Methods," we obtained a small

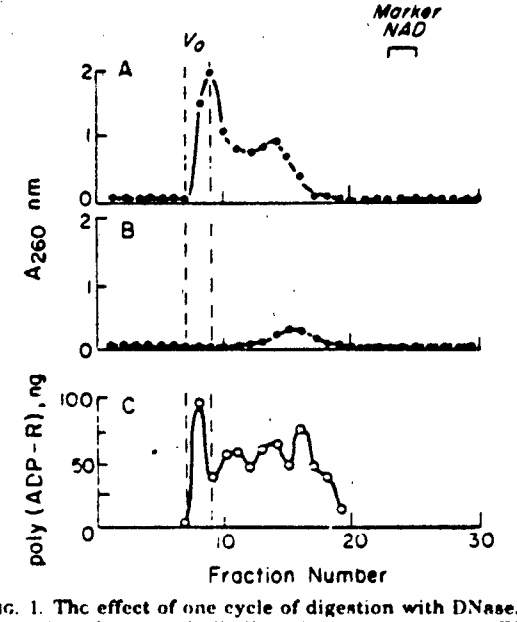


FIG. 1. **The effect of one cycle of digestion with DNase, micrococcal nuclease, and alkaline phosphatase on the UV absorbance (A_{260} nm) and immunochemically detectable polyadenosine diphosphoribose content of a 1 M NaOH digest of rat liver.** Elution patterns are determined by molecular filtration on Sephadex G-50 as described under IID under "Materials and Methods." *A*, molecular profile of DNA as determined by A_{260} nm before digestion with nucleases; *B*, same as *A*, except after one cycle of digestion with nucleases; *C*, macromolecular profile of immunoreactive larger than tetrameric polyadenosine diphosphoribose after one cycle of digestion with nucleases.

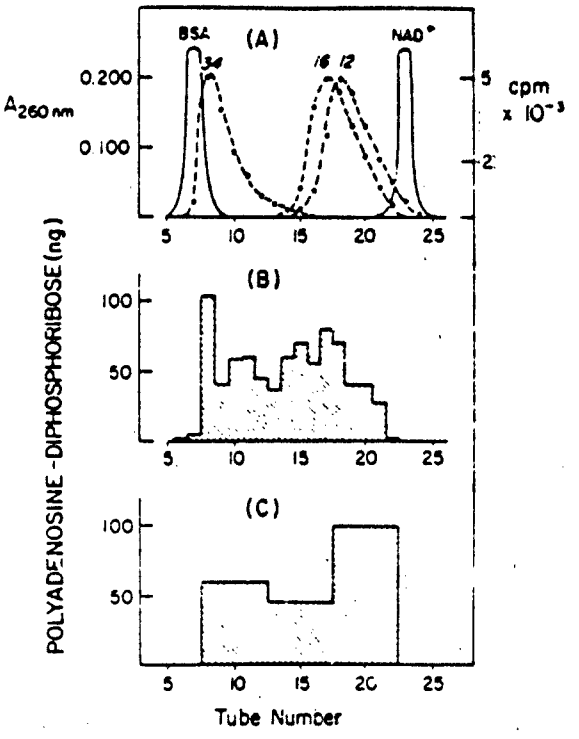


FIG. 2. **Polydispersity of macromolecularly associated immunoreactive polyadenosine diphosphoribose $n > 4$.** *A*, calibration of Sephadex G-50 column with bovine serum albumin (BSA), polyadenosine diphosphoribose containing 34, 16, and 12 ADP-R units, and NAD^+. ——, A_{260}; - - -, ^{14}C counts per min. *B*, macromolecular profile of polyadenosine diphosphoribose isolated from the non-histone fraction of anhydrous rat liver powder as determined by molecular filtration on Sephadex G-50 and radioimmunoassay. *C*, macromolecular profile of histone f1-bound polyadenosine diphosphoribose by the same method as given in *B*. Details are described under IID under "Materials and Methods."

fraction of dilute acid-insoluble rat liver proteins which bound to the dihydroxyboryl resin (about 800 $\mu g/g$ wet weight). The binding of this small amount of protein to the boronate resin implies covalent attachment to polyadenosine diphosphoribose. Subsequent hydrolysis and radioimmunoassay of the polyadenosine diphosphoribose liberated from the protein gave a protein/polymer weight ratio estimate of 400:1. MacGillivray *et al.* (15) have suggested that a sizable fraction of the non-histone proteins covalently bound to polyadenosine diphosphoribose have large molecular weights in excess of 70,000. The polyadenosine diphosphoribose released from protein corresponded to a liver-content of 1,600 ng/g wet weight. This value is within the range of larger than tetrameric polymer content as determined in total non-histone proteins by the methods described in this paper and suggests that the polyadenosine diphosphoribose is covalently protein-bound.

Since our conditions for solubilization of dilute acid-precipitable proteins by 6 M guanidine hydrochloride in 100 mM Tris, pH 8.2, are similar to the conditions used by others for the solubilization of non-histone proteins from nuclei or chromatin (38), it is apparent that the bulk of non-histone proteins were extracted by our method from the 5% perchloric acid precipitate.

It has been recently demonstrated that the polyadenosine diphosphoribose polymerase is located in the internucleosomal regions of chromatin (12, 13). This localization predicts that the enzymatic products should accumulate between nucleosomes and probably modify by as yet unknown mecha-

nisms proteins also localized in the internucleosomal areas. Our results tend to confirm this prediction. Observed phenomena of regulation of DNA synthesis by polyadenosine diphosphoribosylation (39) and an as yet unexplained role of polyadenosine diphosphoribose in differentiation (11, 40, 41) and enzyme induction (42) may be related to a modifying effect of polyadenosine diphosphoribosylation on predominantly non-histone proteins. These proteins are known to play a key role in transcription and its regulation (43). The polydispersity of various histone associated ADP-R polymers that are present in small amounts indicates that the dimer formation of histone f1 (16, 17) is not a unique macromolecular species.

Our work thus far has not included the determination of polyadenosine diphosphoribose $n < 4$. Monomers of ADP-R which are bound to protein would be decomposed to AMP under our conditions. Combined chemical and immunological analyses of all forms of polyadenosine diphosphoribose and the study of their physiological role are the subject of further investigation.

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MOLOGY

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Methods in Enzymology

Volume 66

Vitamins and Coenzymes

Part E

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however, repetition of freezing and thawing usually leads to a considerable loss of the catalytic activity. It is also noteworthy that DNA tends to facilitate the inactivation of enzyme.

[26] Extraction and Quantitative Determination of Larger than Tetrameric Endogenous Polyadenosine Diphosphoribose from Animal Tissues

By TAKEYOSHI MINAGA and ERNEST KUN

Evaluation of the *in vivo* biological role of polyadenosine diphosphoribose in animal organs has thus far been attempted by *in vivo* labeling of the polymer with [^{14}C]ribose.[1] This approach, although clearly indicating the existence of the polymer in rat liver, necessarily depends on its detection by the ^{14}C marker. The determination of the metabolic turnover of polymeric polyadenosine diphosphoribose *in vivo* requires an independent assay procedure for the quantitative determination of the polymer in tissues. Combination of the quantitative assay with tracer techniques is a prerequisite for the study of *in vivo* metabolism of polyadenosine diphosphoribose in normal and abnormal biological states. A selective radioimmunoassay for macromolecular polyadenosine diphosphoribose (larger than tetrameric polymer) has been described which is suitable for the analysis of animal tissues.[2] The method requires the partial purification of nuclei from freeze-dried, rapidly frozen organs with the aid of organic solvents.[2] Although reliable data and enzymically active nuclei can be obtained, the method suffers from the disadvantage that the isolation of nuclei by organic solvents is of low yield and is time-consuming. Modification of the extraction and digestion methods as described here circumvents the necessity to isolate nuclei and enables the accurate determination of polymeric polyadenosine diphosphoribose [$(ADP\text{-}R)_{n>4}$] by the radioimmunoassay[2] in animal tissues. The method is suitable for the intrachromatin localization of the polymer.

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[2] A. M. Ferro, T. Minaga, W. N. Piper, and E. Kun, *Biochim. Biophys. Acta* 519, 291 (1978).

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Method

I. Analysis for Total Poly(ADP-R)$_{n>4}$

(a) Sampling. One to ? g of fresh tissue are freeze-clamped at liquid N_2 temperature and freeze-dried. A stable powder is obtained suitable for storage (at −20°) and subsequent analyses.

(b) Homogenization and Extraction. A weighed sample of the freeze-dried tissue powder is homogenized (ice bath) in 20 volumes of 10% $HClO_4$ (0°). The sediment is separated by centrifugation (4°, 7000 rpm for 10 min), washed twice with 10% $HClO_4$, resedimented, and the supernatants combined. Homogenization and extraction with $HClP_4$ (10%) is advantageous for two reasons: (*a*) it separates soluble nucleotides (e.g., NAD^+); (*b*) it prepares the protein and nucleic acid sediment for efficient subsequent digestion.

(c) Digestion in NaOH. The $HClO_4$ precipitate from (*b*) is suspended in water (1 ml for 200 mg powder) plus an equal volume of 2 *M* NaOH (stir by Vortex); an equal volume (e.g., of the total at this stage) of 1 *M* (final concentration) NaOH is added, and the mixture is digested for 24 hr at 37°.

(d) Enzymic Digestion. The pH of the NaOH digest is adjusted to pH 8.5 (glass electrode) with 3 *M* HCl plus Tris base, and $MgCl_2$ is added to a final concentration of 10 m*M*. To a final concentration of 90 μg/ml DNA-ase, 12 μg/ml micrococcal nuclease and 30 μg/ml of alkaline phosphatase (this may be omitted if termini of polyadenosine diphosphoribose are to be maintained intact) are added; the mixture is incubated for 6 hr at 37°, and then all the nucleases in the same amounts are *again* added before continuing the incubation overnight at 37°. Next morning EDTA is added to a final concentration of 20 m*M*, SDS to 0.25%, and proteinase K (1.7 mg/ml final concentration) and the solution incubated with vigorous shaking for 12 hr at 37°.

Undigested material is spun down and the clear supernatant fluid saved. The insoluble material is resuspended in 500 m*M* Tris-HCl, pH 8.5 (5 ml buffer for 200 mg original $HClO_4$ precipitate), proteinase K (1 mg/ml) added, and the mixture redigested for 12 hr at 37° with agitation. The procedure (i.e., sedimentation of undigested material, saving and combining of supernatant, and redigestion with proteinase K) is repeated 5 times in succession. The final insoluble pellet contains only 0.2% of ^{14}C-labeled polymers and is discarded.

(e) Extraction with Phenol. The combined soluble digest is extracted with water-saturated phenol (10 ml phenol per 30 ml extract), and layers are separated by centrifugation (3000 rpm for 10 min). The phenol phase is back-extracted with water (5 ml per above stated volume). The com-

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total at this stage) of 1 *M*
mixture is digested for 24

H digest is adjusted to pH
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concentration of 90 μg/ml
30 μg/ml of alkaline phos-
denosine diphosphoribose
cture is incubated for 6 hr
amounts are *again* added
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bined aqueous fractions are re-extracted with 10 ml (per 30 ml digest) water-saturated phenol, washed with H_2O, and the combined aqueous fractions re-extracted with water-saturated diethyl ether (100 ml per 30 ml original digest). The ether extract is back extracted with water. The combined water extracts contain 85–97% of poly(ADP-R) as tested by the recovery of added ^{14}C-labeled polymer to the enzymic digest.

(f) Dialysis and Freeze-Drying. The aqueous extract is extensively dialyzed (24 hr with 5 changes of water) and then freeze-dried. This extract still contains ultraviolet-absorbing material which cannot be accounted for as poly(ADP-R), but this does not interfere with the radioimmunoassay.[3] Redigestion with the nuclease cycle and further digestion with α-amylase can remove non-poly(ADP-R) contaminants if the purpose of isolation is to obtain radiochemically and immunochemically pure polymer (i.e., in [^{14}C]ribose *in vivo* labeling experiments). However, for quantitative radioimmunoassay by the glass-fiber technique, the above extract is satisfactory.

II. Subfractionation into Histone and Nonhistone Associated Poly(ADP-R)$_{n>4}$

(*a*) Extraction of histone H1 from the original freeze-dried powder is readily accomplished by 5% $HClO_4$ (2 g powder plus 10 ml 5% $HClO_4$, homogenize at 0°, then stir for 30 min at 5°, centrifuge, repeat 3 times, combine supernatants). To the combined $HClO_4$ extracts, trichloracetic acid is added to a final concentration of 20% and 6 mg bovine serum albumin are added as a carrier at 0°. The precipitate is collected, redissolved in a small volume of water, dialyzed, and digested as described in I.

(*b*) Extraction of total histone fraction is carried out with 0.25 *M* HCl from the original freeze-dried powder (2 g powder plus 50 ml 0.25 *M* HCl, homogenize, then stir for 30 min at 5°, repeat 3 times, combine

POLY(ADP-R)$_{n>4}$ CONTENT OF 1 g FREEZE-DRIED POWDER OF RAT LIVER

No.	Fraction	ng poly(ADP-r)$_{n>4}$ per 1 g dry powder
1	Total powder	8739.3
2	Total histone-associated (ADP-R)$_{n>4}$	166.0
3	Histone H1-associated (ADP-R)$_{n>4}$	18.8

supernatants). The combined HCl extract is dialyzed, freeze-dried, and then digested as in I.

A representative analysis is shown in the table.

Summary

The validity of the extraction technique was tested by recovery of added ^{14}C-labeled polymer. Recovery was always close to 90%. It is apparent that the predominant chromatin fraction, containing more than 99% of $(ADP\text{-}R)_{n>4}$, is the nonhistone fraction.

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[27] Covalent Modification of Proteins by Metabolites of NAD$^+$

By ERNEST KUN

Polymeric and oligomeric products of the adenosine diphosphoribose moiety of NAD$^+$ are known to be formed in the chromatin of eukaryotic cells.[1,2] Although the binding of $(ADP\text{-}R)_n$ to macromolecules of chromatin is assumed to be at a template site of the synthetase system, the mechanism of this binding, or the transfer of $(ADP\text{-}R)_n$ fragments from the polymer to other protein acceptors (histone and nonhistone proteins) is as yet unclear. The covalent modification of proteins of chromatin by ADP-R transfer from poly(ADP-R) cannot be described in uniform molecular terms because presumably N,O-ribosidic bonds and possibly ester-phosphate bonds have been reported to exist between ADP-R residues and proteins.[1]

This chapter is restricted to the description of two types of ADP-R-protein interactions: the enzymic transfer of ADP-R from NAD$^+$ to a

[1] H. Hilz and P. Stone, *Rev. Physiol. Biochem. Pharmacol.* **76**, 1 (1976).
[2] O. Hayaishi and K. Ueda, *Annu. Rev. Biochem.* **46**, 95 (1977).

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NOVEL ADP-RIBOSYLATIONS OF REGULATORY ENZYMES AND PROTEINS

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S. Hayaishi, Sugimura, eds. Novel ADP Ribosylations of Regulatory Enzymes and Proteins

SUBNUCLEAR LOCALIZATION OF POLY(ADP-RIBOSYLATED) PROTEINS

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INTRODUCTION

Covalent modification of macromolecules constitutes a highly versatile and specific chemical regulatory device that plays a prominent role in diverse cellular processes.[1] The advantage of this type of control mechanism over direct kinetic regulation of enzymatic reactions is the extraordinary specificity inherent to the enzyme system that catalyzes the covalent modification of macromolecules. As is the case in many complex biochemical hierarchies, the regulatory implications of macromolecular modification cannot be predicted solely on the basis of the catalytic properties of its constitutive enzymes. Undoubtedly among the presently unexplained functions of eukaryotic chromatin are processes which determine differentiation and cell division with its anomalies. An understanding of any of these complex events requires the detailed knowledge of the regulatory effects of macromolecules of chromatin on - as yet incompletely understood - enzymatic components of DNA and RNA metabolism. The recognition of discrete nucleosomal components of chromatin represents probably the most significant recent achievement in this field, although internal architectural details of nucleosomes still require clarification. As emphasized by Felsenfeld,[2] the regulation of chromatin must involve also proteins other than the known components of nucleosomes (eg. nonhistone proteins). The question can be raised whether or not the customary methods of isolation of nuclei result in a loss of macromolecular nuclear components that are significant in the in vivo regulation of chromatin. It follows that the already numerous reports professing to explain the regulatory role of the poly(ADP-R) system in chromatin function may be premature because the hypothetical models chosen are derived from as yet incomplete experimental information.

Notable advances were made in several laboratories with highly purified poly(ADP-R) polymerases.[3,4,5,6] The results show that an apparently homogeneous protein catalyzes the in vitro synthesis of very short (ADP-R) oligomers, which can be elongated when N_1-(ADP-R) Schiff base is present.[3,7] A specific double stranded DNA also participates as a co-factor.[5]

a. Recipient of the Research Career Award of the U.S.P.H.
b. Recipient of a N.S.E.R.C. (Canada) Fellowship

We have chosen to investigate the biology of poly(ADP-R) by comparing *in vivo* and *in vitro* subnuclear protein associations of (ADP-R) polymers in view of the enzymologically unresolved complexity of the mechanism of poly (ADP-R) formation and the previously mentioned uncertainties in our knowledge of chromatin structure.

MATERIALS AND METHODS

Male Sprague Dawley rats (200 g body weight) were deprived of food for 24 hours prior to the removal of livers for analysis. Most of the studies concerned with the subnuclear distribution of poly(ADP-R) and with *in vivo* labelling were done with small rodents. Methods of isolation and fractionation of protein poly(ADP-R) adducts, the techniques of radioimmunoassay and identification of (ADP-R) oligomers are described elsewhere.[8,9]

Dimethylnitrosamine (99% Aldrich Chemicals) treatment of Golden Syrian hamsters was carried out by Dr. C.L. Gaworski, at the University of California, Toxic Hazards Research Unit Overlook Branch, Dayton (Ohio, 45431) according to a published scheme.[10] Treatment schedule was as follows. Hamsters, 4 months old (50 animals/group), were injected intraperitoneally once a week with a 1% aqueous solution of dimethylnitrosamine, 0.5 mg per animal for 4 weeks. The treatment was then terminated and biochemical analyses performed. Controls received H_2O only. Histological examination for tumor cell development was 100% negative at this stage.

The isolation of protein (ADP-R) adducts was carried out on boronate affinity columns. These columns were synthesized by covalent attachment of m-amino phenylboronic acid to Biogel P-300 (50-100 mesh) via a glutarylhydrazide spacer arm. Quantitative binding of protein poly(ADP-R) covalent complexes to the affinity column was achieved at pH 8.2 and elution of the complex was carried out at pH 4-6 at 25°C.

RESULTS

Quantitative assessment of poly(ADP-R) in cellular and subcellular systems has been usually based on the radiochemical assay of the acid precipitable or electrophoretically or chromatographically separated polymer. Since no specific precursors for poly(ADP-R) exist,[11] ^{14}C-ribose or ^{32}P are suitable *in vivo* labels, provided appropriate purification of poly(ADP-R) precedes the determination of radioactivity. The *in vivo* labelling of poly(ADP-R) by adenine proved to be unsuitable in our hands. The quantitative determination of poly (ADP-R) by pulse labelling presupposes that the specific activities of all

labelled species of poly(ADP-R) are the same, otherwise uneven labelling necessarily falsifies results. This is the case illustrated in the following experiment.* Infant rats (50 g weight) were injected with 20 μCi D(1-^{14}C) ribose/rat and poly(ADP-R) of liver isolated by the conservative technique developed in our laboratory.[8] Molecular filtration of the polymer was carried out using poly(ADP-R) isolated over a period of 30 to 150 minutes after labelling with ribose. The results indicated the appearance of two major macromolecular species, I = $(ADP\text{-}R)_{n=30}$ and II = $(ADP\text{-}R)_{n=12}$ as shown in Fig. 1.

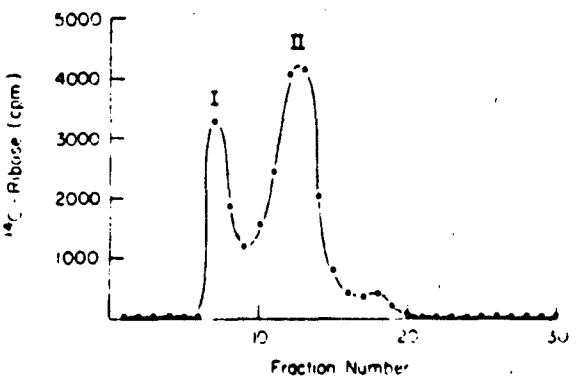

Fig. 1. ^{14}C-ribose content of poly(ADP-R) fractionated on Sephadex G-50.

When the percentage of total ^{14}C-ribose labelled (ADP-R) in the two macromolecular fractions is determined at 30 minute intervals, utilizing 2 rats for each timed assay, a characteristic fluctuation of specific ^{14}C-ribose distribution is observed (Fig. 2). It is apparent that $(ADP\text{-}R)_n$ in both macromolecular fractions exhibits metabolic fluctuations consistent with apparent transfer of (ADP-R) units between oligomers of different sizes. If this macromolecular metabolic flux of $(ADP\text{-}R)_n$ were ignored, false polymer content would be calculated on the basis of ^{14}C-ribose assay alone, since the existence of various pools of poly(ADP-R) is possible. The apparent $t_{1/2}$ of labelling of $(ADP\text{-}R)_{n=30}$ corresponds to the $t_{1/2}$ of labelling of NAD$^+$, ie. 3.1 to 2.7 hours respectively.

Due to the uncertainties of poly(ADP-R) assay based on precursor incorporation, we adopted a quantitative radioimmunoassay for $poly(ADP\text{-}R)_{n>4}$[9] for the determination of covalently bound poly(ADP-R) to proteins of chromatin.

* Minuga, T. and Kun, E., unpublished experiment, 1977.

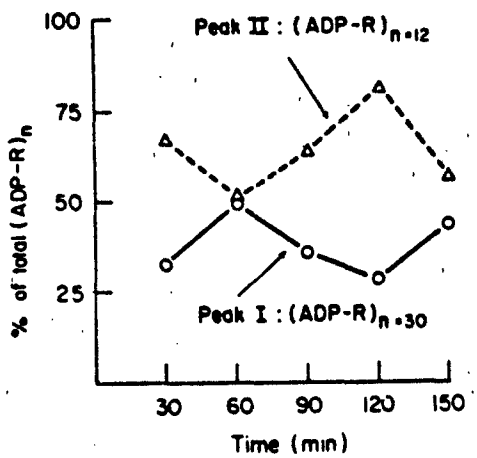

Fig. 2. Time dependent fluctuation of ^{14}C-ribose in the two macromolecular fractions of poly(ADP-R) as shown in Fig. 1.

When nuclear proteins are separated by their solubility in mineral acids, histone-associated (ADP-R) polymers are readily extracted, and because of this obvious technical convenience, most work on ADP-ribosylation of proteins has been confined to studies on (ADP-R) histone adducts.

TABLE 1

POLYADENOSINE DIPHOSPHORIBOSE IN ACID EXTRACTS AND SEDIMENTS OF RAT LIVER POWDER

Extracting Acid	Polyadenosine Diphosphoribose$_{n>4}$, ng/g dry powder		
	Supernatant	Sediment	Total
5% $HClO_4$	90.0	9,600	9,690
0.25 N HCl	166.0	8,573	8,739

As presented in Table 1 (cf. 8), more than 99% of immunochemically reactive n > 4 (ADP-R) polymers are found in the nonhistone protein sediment after acid extraction. It should be noted that although electrophoretic mobility of histones (notably H_1) is modified by ADP-ribosylation,[7] solubility properties

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TABLE 2

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in acids or organic solvents are not altered. Apparently in histones only a small number of basic groups are modified by ADP-ribosylation. This is in sharp contrast to the behavior of DNA-histone complexes, which are known to be acid precipitable.

Subfractionation of acid extractable protein $(ADP\text{-}R)_{n>4}$ adducts by chromatographic and organic solvent extraction procedures (cf. 8), showed that soluble nonhistone protein components are also present in this fraction containing (ADP-R) oligomers where $n>4$. This is illustrated in Table 2 (cf. 8).

TABLE 2

POLYADENOSINE DIPHOSPHORIBOSE CONTENT OF ACID SOLUBLE TISSUE FRACTIONS OF RAT LIVER POWDER

Method of Extraction and Fractionation	Polyadenosine Diphosphoribose$_{n>4}$ ng/g dry powder
5% $HClO_4$ Extract	96.0
20% Trichloracetic Acid Supernatant of $HClO_4$ Extract	80.0
20% Trichloracetic Acid Precipitate of $HClO_4$ Extract	
Nonhistone Peptides	...
Purified Histone H_1	11.5
B	
0.25 N HCl Extract	166.0
Nonhistone Fraction	90.0
Histone H_1	16.0
Histone H_2 + H_3 Fraction	60.0

The quantitative distribution of $(ADP\text{-}R)_{n>4}$ in histones isolated by standard chromatographic and organic solvent techniques in nanograms per gram dry liver was H_1=21.7, H_{2A}=12.2, H_{2B}=2..2, H_3=18.7. The micromolecular dispersity of nonhistone protein bound (B) and histone H_1 bound (C) poly(ADP-R) is shown in Fig. 3. Polymers varying from 12 to 34 (ADP-R) are found in all fractions.

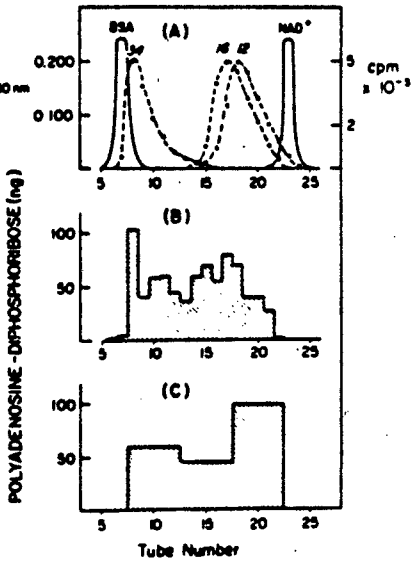

Fig. 3. Macromolecular profile of poly(ADP-R) isolated from nonhistone protein (B) and Histone H_1 (C).

We have further investigated the as yet unsolved question of whether there are free $(ADP\text{-}R)_n$ oligomers in nuclei, or whether all (ADP-R) polymers are protein bound. Affinity chromatography on acrylamide-dihydroxyboryl columns proved to be a suitable technique to settle this question. We find that all immunochemically detectable $(ADP\text{-}R)_{n>4}$ as assayed in whole liver tissue can be isolated as $(ADP\text{-}R)_{n>4}$ protein adducts, that is in vivo, all $(ADP\text{-}R)_{n>4}$ polymers are covalently bound. Incubation of ^{14}C-NAD^+ with isolated nuclei in the absence of phenylmethylsulfonylfluoride (PMSF) yielded significant amounts of free (ADP-R) oligomers, whereas the presence of the protease inhibitor completely abolished the appearance of free (ADP-R) oligomers. It is evident that free poly(ADP-R) is an artefact due to the interference of proteases. As shown in Fig. 4, the boronate affinity column quantitatively isolates the (ADP-R) protein adducts that are formed during in vitro incubation of liver nuclei with NAD^+ in the presence of protease inhibitor. The macromolecular fraction appears in the void volume of Sephadex G-50 column. When the inhibitor is omitted or the protein (ADP-R) adducts are decomposed in 1N NaOH, free (ADP-R) oligomers appear. (ADP-R) oligomers n=12 and NAD^+ were used as markers in this experiment.

Fig. 4. Mole
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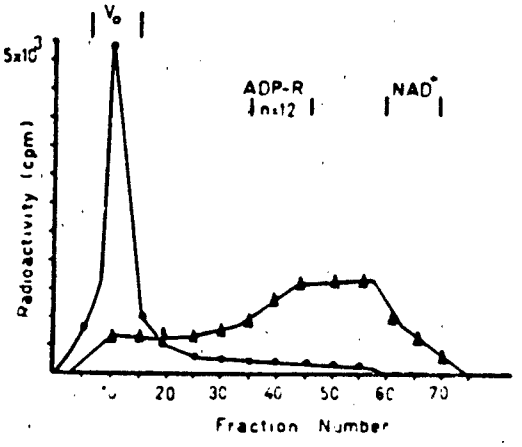

Fig. 4. Molecular filtration of poly(ADP-R) (isolated by borenate affinity chromatography) on Sephadex G-50. V_o = protein-poly(ADP-R) adduct extracted from nuclei incubated in the presence of PMSF. The broad peak shows free $(ADP-R)_n$ released by base treatment or formed in the absence of protease inhibitor.

The identity of the protein bound (ADP-R) oligomer was established by its resistance to RNA-ase and DNA-ase, but subsequent digestion by snake venom phosphodiesterase.

The dihydroxyboryl affinity chromatography technique, illustrated in Fig. 5, enables a direct isolation of the natural products of the poly(ADP-R) polymerase enzyme system. Although we are in the process of perfecting this technique, its usefulness was tested even in its present stage of development. Since the predominant target proteins of poly(ADP) ribosylation are nonhistone proteins,[8] that are known[12] to play a special role in the regulation of chromatin, we determined their poly(ADP) ribosylation in nuclei isolated from livers of normal and pre-cancerous hamsters. Enzymatic assay of poly(ADP-R) polymerase at two protein concentrations revealed a significant decrease of enzymatic activity in nuclei isolated from livers of dimethylnitrosamine treated hamsters. In agreement with the enzymatic assay, the quantity of (ADP-R) protein adducts isolated from nitrosamine treated hamsters by the affinity column method, also diminished significantly in nuclei incubated in vitro with NAD^+.

It is predictable that analyses of an entire population of nuclei prepared from the heterogeneous cellular mass of a liver may be severely falsified, since it is known that the carcinogenic action of dimethylnitrosamine is confined to

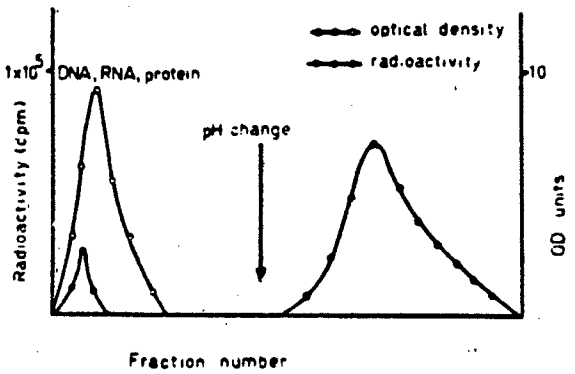

Fig. 5. Elution profile of poly(ADP-R) protein adducts from a boronate affinity column run in 6M guanidine-HCl. The pH change was from 8.2 to 6.

a relatively small number of liver cells. It follows that a large effect on relatively few nuclei could be diluted by the predominance of normal nuclei.

Despite this unfavorable statistical condition, a 38% decrease of (ADP-R) protein formation (Fig. 6, C) and a 22% decrease of apparent polymerase activity (Fig. 6, A,B) were detectable in livers of dimethylnitrosamine treated hamsters; therefore a far larger quantitative and perhaps qualitative difference may actually exist in the selected number of pre-cancerous liver cells in vivo. We are presently developing an isopicnic centrifugation technique capable of separating various populations of liver cell nuclei. It is anticipated that more detailed studies of various populations of liver cell nuclei will provide further more specific information.

Fig. 6. Poly(AD
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or B)).

DISCUSSION

Experimental results illustrate the importance of assessing the quantities of (ADP-R) polymers by a direct analytical method, that is independent of the radiochemical detection of a labelled precursor incorporated into the polymer. Basic nuclear proteins assume a relatively minor role (less than 1%) in serving as $(ADP\text{-}R)_{n>4}$ acceptors (Tables 1, 2). This fact alone does not argue against the possible significance of ADP ribosylation of histones in the regulation of chromatin; however, a singularly important modification of polynucleosomal chromatin structure by the modification of H_1 alone seems less convincing in view of the fact that in vivo, all species of histones are nearly equally polyadenosinediphosphoribosylated. The preponderance of nonhistone proteins

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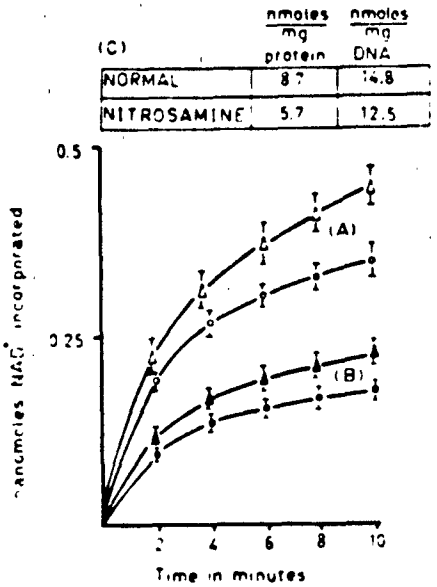

Fig. 5. Poly(ADP-R) polymerase activities of nuclei isolated from normal and pre-cancerous hamsters. (A = 0.3 mg protein/200 µl test system, B = 0.12 mg/200 µl test system) ^{14}C-NAD^+ = 2.8×10^7 dpm, NAD^+ = 0.5 mM, 0.5 mM EDTA, 100 mM Tris-HCl (Ph 8.0), 2 mM dithiothreitol, 6 mM KF, 20 mM $MgCl_2$, 1 mM PMSF, T = 30°C. (C = the quantity of poly(ADP-R) protein adducts isolated by affinity chromatography from nuclei (28 mg protein) incubated for 30 minutes, (as in A or B)).

which serve as $(ADP\text{-}R)_{n \geq 4}$ acceptors, introduces the possibility that the $NAD^+ \longrightarrow$ poly(ADP-R) pathway may profoundly modify proteins of unknown function that possess the unique property of forming immunologically tissue specific complexes with homologous DNA.[15]

The significant decrease of poly(ADP) ribosylation of nuclear proteins by nuclei isolated from pre-cancerous hamster livers tends to suggest that further analysis of this phenomenon may lead to a biochemically definable system that is related to de-differentiation. Since histological examination of pre-cancerous livers revealed no traces of necrosis, the enzymatic and chemical analyses are not falsified by a non-specific decrease of cellular proteins. This problem as well as the physiologically meaningful de-repression of cardiac protein synthesis in vivo following the inhibition of $(ADP\text{-}R)_n$ synthesis[16] are being further studied.

ACKNOWLEDGEMENTS

This work was supported by grants from the American Cancer Society (BC-304) and the United States Air Force Office of Scientific Research (AFOSR-78-3698A).

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DISCUSSION

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DISCUSSION

DR. JACOBSON: Concerning the data on the last slide, was that poly(ADP-ribose) content?

DR. KUN: That was the synthesis of a poly(ADP-ribose)-protein complex in a large incubation.

DR. GILL: On one of your slides, you indicated that you had detected free polymers in vivo. What is your evidence that what you got was free polymer?

DR. KUN: We identified it by enzymic digestion.

DR. GILL: How does that show that it's free?

DR. KUN: There's no protein in it.

DR. GILL: You mean you add enzyme to it and then show there's no protein there?

DR. KUN: No, we isolated the product.

DR. GILL: You assume that these are peptides?

DR. KUN: Yes.

DR. HOGEBOOM: Did you give the data in terms of mg poly(ADPR)/mg DNA?

DR. KUN: Yes, approximately 500 ng/mg DNA.

DR. ALAN: There are chain length effects of antibody binding that must be corrected for and these corrections may be important.

DR. KUN: There is some disagreement about the antibody we use and that of your laboratory. Our antibody doesn't see any differences in chain lengths beyond 2.

DR. MIAN: Have you fractionated the naturally occurring poly(ADP-ribose) by gels

DR. KUN: Yes.

DR. [illegible] KAY: If you have a chain that is twenty residues long, won't it bind more antibodies than the chain that is four residues long.

DR. KUN: No, because the antibody has a preference, etc.

DR. KAY-FLYNN: I would expect it to bind more.

DR. [illegible]: I would like to comment on this point. The standardization of our method was done with defined chain lengths produced by phosphodiesterase. Is it could be that your antibody really recognizes not the usual poly(ADP-ribose) structure but a structure which is produced by a phosphodiesterase, such a ribosylribosemate derivative. Can you exclude that possibility.

DR. KUN: The antibody reacts with polymers that have not been treated with phosphodiesterase.

DR. HILL: If you cannot differentiate between a chain of 30 and a chain of 5, how can you make the method quantitative?

DR. KUN: We counting chains.

DR. KAYSER: Is it possible that your antibody preparation contains histones that don't show poly ADPR binding activity?

DR. KUN: We don't think that this is the case since we have purified it extensively.

From Gene to Protein: Information Transfer in Normal and Abnormal Cells, edited by Thomas R. Russell, Kelly Brew, Harvey Faber, and Julius Schultz; published by Academic Press, New York, 1979.

FRIDAY, SEPTEMBER 26th

2C CHEMICAL ASPECTS OF BIOLOGICAL RECOGNITION
CYCLIC AMP, CYCLIC GMP, ENZYMES AND OTHER MEDIATORS OF HORMONAL SIGNALS IN BIO-DEVELOPMENT

Chairmen: O.Gašić (Yugoslavia), M.S.Kus (Turkey)

9.30-9.45 p. 58 — REGULATION OF NON-HISTONE CHROMOSOMAL PROTEINS BY ENZYMATIC ADP-RIBOSYLATION: PROBABLE SITES OF METABOLIC EPIGENETIC SIGNAL TRANSFER, AND DEVELOPMENTAL CONTROL
E.Kun (U.S.A.)

9.45-10.00 p. 23 — CHANGES IN THE ENZYME ACTIVITIES IN TISSUES OF URBASON TREATED GUINEA PIGS AT DIFFERENT DEVELOPMENTAL STAGES
P.G.Cholakova-Boyadjieva (Bulgaria)

10.00-10.15 p. 51 — STABILITY OF ACID PHOSPHATASE IN PRESENCE OF ANTISERUM
D.K.Kapoor & G.S.Gupta (India)

10.15-10.30 p.59 — KINETIC PROPERTIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN THE HUMAN NORMAL AND SENILE CATARACTOUS LENSES
M.S.Kus &K.Mergen (Turkey)

10.30-10.45 p. 35 — THE DETERMINATION OF α- AND β -AMYLASE ACTIVITY IN SOME GENTYPES AND HYBRIDS OF WHEAT
O.Gašić, D.Štajner & M.Kraljević-Balalić (Yugoslavia)

10.45-11.00 p. 46 — TO THE ADENYLATE CYCLASE DETERMINATION IN HIGHER PLANTS
M.A.Ivanova, E.P.Fedenko & N.G.Doman (U.S.S.R.)

11.00-11.30 — BREAK

FRIDAY, SEPTEMBER 26th

3 MATHEMATICAL APPROACHES, MODELS AND HYPOTHESES FOR STUDYING BIOLOGICAL RECOGNITION. OSCILLATORY PHENOMENA IN THE BIO-RECOGNITION PROCESS

Chairmen: E.Kőrös (Hungary), D.Chernavskii (U.S.S.R.)

11.30-12.00 p. 26 — THE CONCEPT OF INFORMATION IN BIOLOGY. APPROACH OF A MATHEMATICAL THEORY OF SIGNIFICATION
F.Collot (France)

12.00-12.15 p. 7 — OSCILLATORY BEHAVIOUR OF LUTEINIZING HORMONE REGULATION
A.S.Alexandrov, J.G.Vassileva-Popova & M.D.Kotarov (Bulgaria)

12.15-12.30 p. 5 — CAN CHEMICAL OSCILLATORS SERVE AS MODELS FOR OSCILLATORY BIOLOGICAL PROCESSES?
E.Korös (Hungary)

Societa Italiana di Biochimica
26 Congresso Nazionale
September 24-26, 1980
Bologna

MERCOLEDI' 24 SETTEMBRE

9.00 — **INAUGURAZIONE DEL CONGRESSO**
Sala « Italia »

9.45 — Conferenza d'apertura
G. Blobel (Rockefeller University, New York)
« Regulation of intracellular protein traffic »

10.30 — Intervallo

10.45 — Discussione
B. Cancedda (Napoli)
« Sintesi e maturazione delle glicoproteine del Virus Sindbis », intervento programmato

11.30 — A. Bargellesi Severi (Genova)
« Anticorpi monoclonali: una nuova tecnica per produrre reagenti sierologici »

12.30 — Fine della sessione

12.30 - 15.30 — ESPOSIZIONE E DISCUSSIONE DEI POSTERS (*)
Sala Posters

15.30 — TAVOLA ROTONDA: « Modificazioni post-traduzionali delle proteine »
Sala « Italia »

Organizzatori: E. Leone, L. Pinna, C. A. Rossi

L. Engström (University of Uppsala, Uppsala)
« Regulation of enzyme activity through cyclic AMP-stimulated phosphorylation with special reference to liver pyruvate kinase »

L. Pinna, F. Meggio e A. Donella-Deana (Padova)
« Riconoscimento dei siti di fosforilazione da parte delle protein-cinasi »

E. Kun (University of California, San Francisco)
« Poly ADPR metabolism in the precancerous state »

S. Pontremoli e E. Melloni (Genova)
« Regolazione della fruttoso 1,6 bisfosfatasi mediante 'proteolisi limitata' ed interazioni proteina-proteina »

17.30 — Discussione ed interventi programmati (**)

19.00 — Saluto del Sindaco di Bologna ai Congressisti e rinfresco offerto dal Comune nelle sale delle Collezioni Comunali d'arte di Palazzo d'Accursio
Palazzo d'Accursio

GIOVEDI' 25 SETTEMBRE

9.00 — **TAVOLA ROTONDA « Meccanismi di Catalisi »**
Sala « Italia »

Organizzatori: E. Antonini, B. Curti, E. Grazi

A. Colosimo, M. Brunori, E. Antonini (Roma)
« Meccanismi catalitici delle ossidasi »

E. Grazi (Ferrara)
« Influenza degli altri enzimi glicolitici sullo stato stazionario degli intermedi catalitici dell'aldolasi muscolare »

G. L. Rossi (Parma)
« Gliceraldeide 3 fosfato deidrogenasi »

G. Zanetti (Milano)
« Meccanismo catalitico delle flavo proteine deidrogenasi NADP dipendenti »

11.00 - 11.15 — Intervallo

11.15 - 12.30 — Discussione ed interventi programmati (**)

12.30 - 15.30 — ESPOSIZIONE E DISCUSSIONE DEI POSTERS (*)
Sala Posters

15.30 - 17.30 — DISCUSSIONE COORDINATA DI POSTERS

Sala azzurra — C. Ricci e G. Ronca organizzeranno la discussione di postere riguardanti temi di « Enzimologia »

Sala verde — P. L. Ipata e F. Salvatore organizzeranno la discussione di postere riguardanti temi riferibili a « Acidi nucleici e metabolismo nucleotidico »

Sala rossa — D. Cavallini e V. Zappia organizzeranno la discussione dei postere inerenti al tema « Biochimica dello zolfo e dei composti solforati »

Gli organizzatori di queste discussioni solleciteranno direttamente gli autori dei « Posters » pertinenti a dare il loro concorso.

17.30 — Assemblea generale dei Soci SIB
Sala « Italia »

19.00 - 21.00 — Visita serale guidata alla Pinacoteca Nazionale di Bologna

9.00 — **TAVOLA ROTONDA**
Sala « Italia » — Organizzatori: C. Gr

P. Zahler e H. Sigris
« Hydrophobic labelli

L. M. V. van Golde
« Dynamics of phos
brane: metabolic as

L. Binaglia, R. Rebo
« I digliceridi di me

C. Gregolin, F. Urs
« Perossidazione e
lipo-proteine »

11.00 - 11.15 — Intervallo

11.15 - 12.30 — Discussione ed inte

12.30 - 15.30 — ESPOSIZIONE E DI

15.30 — SESSIONE INTERDI
Sala « Italia » — miocardio « organizz

Relatori:

A. L. Slug (NIH, M
« Metabolic change

E. Carafoli (Politecn
« Ruolo del Ca++
cardica »

17.15 — Discussione

O. Vialetti (Cattedr
Parma)
« Protezione del mi
boliche », intervento

« POSTERS »

(*) I postere hanno le seguenti
90 cm di larghezza. Potranno e
del giorno stabilito e restare
Gli autori del postere contra
pregati di essere disponibili
alle 14.00 quelli, contraddistint
alle 15.30.

« INTERVENTI PROGRAMMATI »

(**) Gli organizzatori delle tavole
autori del postere più pertine

International Titisee Conference:

"Metabolic Interconversion of Enzymes"
October 2 - 4, 1980

NOVEL ASPECTS OF REGULATION

Chair: E. E. Snell; Rapporteur: C. Gancedo

9:00 - 9:35	E. R. Stadtman: Involvement of Adenylylation and NADPH-P_{450}-Linked Mixed Function Oxidation in Regulating the Proteolytic Degradation of Glutamine Synthetase in E. coli
9:35 - 10:05	H. Hilz: ADP-Ribosylation and Poly ADP-Ribosylation of Nuclear Proteins
10:05 - 10:40	E. Kun: ADP-Ribosylation of Actin and Other Non-Histone Proteins During Normal and Abnormal Growth Conditions
10:40 - 11:00	E. Leone: ADP-Ribosylation of Ribonucleases
11:00 - 11:30	Coffee Break

PART 1: PROGRESS REPORT (PERIOD: DECEMBER 1978 TO FEBRUARY 1980)

A. Published results. The purpose of this work was not only to include all molecular species of $(ADP\text{-}R)_n$ in the method of isolation and analysis of poly ADP-R from small tissue samples but also to devise a technique that permits the isolation of poly ADP-R and ADP-R protein adducts, which are the actual products of enzymatic polymer formation. Both approaches were successful and it was shown by immunochemical and later by chemical methods that the predominant poly ADP-R acceptor proteins are non-histone proteins, providing the critical experimental basis for further work. It was also shown by a specially developed boronate-affinity column chromatography which selectively isolates ADP-R and poly ADP-R protein adducts that no free poly ADP-R exists in cells under physiological conditions and previously reported "free poly ADP-R fragments" found in tissues are an artifact of faulty isolation methods. These results were published (1,2) and some results obtained by the boronate affinity method are shown in Fig. 4 and Fig. 5 of ref. 2 and ref. 1. As an approach to the in vivo biological role of poly ADP-R in normal rats and hamsters ^{14}C-ribose labeling kinetics was performed and two main rapidly labeled molecular species of liver poly ADP-R were identified (Fig. 1 of ref. 2). A time dependent variation in macromolecular spec. act. (^{14}C-ribose) was demonstrated (Fig. 2 of ref. 2), illustrating the dynamic state of the polymer. The $t_{\frac{1}{2}}$ of in vivo ribose labeling of both NAD^+ and poly ADP-R were found to be between 2.7 - 3.1 hours; thus a typical precursor-product relationship between NAD^+ and poly ADP-R was established in vivo.

The biological variation of poly ADP-R metabolism during early precancerous state (Fig. 6 of ref. 2) was studied by first determining the rates of enzymatic elongation of poly ADP-R sites in isolated chromatin preparations from normal and carcinogen treated hamster livers. A relatively small (25-32%) but statistically significant decrease in the rates of labeled ADP-R incorporation was recorded in chromatin of hamster treated for one month. As pointed out in ref. 2, it is expected that isolated subpopulations of liver cells are likely to exhibit a much larger response than the average measured in a mixed nuclear population. This problem is presently pursued. In livers of hamsters treated with the hepatocarcinogen for 2 months, the above phenomenon (shown in Fig. 6 of ref.2) was reversed; thus a small increase in rates of ADP-R incorporation (elongation) occurred. Present results suggest that the apparent reversal phenomenon is real, and may indicate a new observation: a time dependent response due to the prolonged effect of the hepatocarcinogen. This question will be further studied.

B. Unpublished experiments. In further work radiochemical and chemical techniques were combined. Chemical methods include the isolation of protein ADP-R adducts by affinity chromatography, cleavage of ADP-R-protein bonds in alkali (0.1 N NaOH) followed by reisolation of free $(ADP\text{-}R)_n$ oligomers on a second boronate affinity column, and after concentration (freeze drying) chemical or enzymatic degradation of the free polymer to characteristic nucleotides that are quantitatively determined by HPLC separation, and UV analysis of specific radioactivities of ADP-R oligomers. It also allows the subsequent two-dimensional electrofocus-electrophoretic separation of de-ADP-ribosylated proteins, that is the identification of proteins which were isolated by virtue of containing covalently bound poly ADP-R.

Summary of in vivo studies. Preliminary data (Table I, Part 2) are consistent with a significant perturbation of NAD^+ → poly ADP-R metabolism in the early precancerous state. The large increase in poly ADP-R content after one month treatment with the hepatocarcinogen followed by a relative decrease in content and simultaneous increase in specific radioactivities of both NAD^+ and poly ADP-R indicates an increased flux throught the NAD^+ → poly ADP-R pathway in the precancerous state. This in vivo kinetic phenomenon coincides with a significant increase in the rate of ^3H-thymidine incorporation into DNA in vivo (pulse labeling with 200 μCi/100 g body weight ^3H thymidine; analysis 18 hours later). The DNA content of precancerous hamster livers was 3.0 mg/1 g (after treatment with the hepatocarcinogen for two months) and in controls was 2.6 mg. The specific radioactivity of DNA is 70,290 dpm/mg DNA in precancerous and 19,380 dpm/mg DNA in controls.

Alkaline sucrose density gradient ultracentrifugal analysis (Fig. 1) shows that there was an increased labeling of all populations of single stranded DNA but a progressively larger labeling of DNA species with short chain length (top of gradient). This labeling pattern can be explained by the increased rates of DNA biosynthesis as observed in vivo. It would be expected that growing chains of single stranded DNA at templates would be progressively more labeled by ^3H-thymidine under these circumstances; therefore a preferential increase of ^3H counts in DNA populations of smaller molecular weight is predictable.*

In vivo labeling of the protein moiety of poly ADP-R-protein adducts by the same dose of ^3H-leucine that significantly labels tissue proteins 30 minutes after i.p. injection (cf. 3) did not introduce detectable ^3H, suggesting a significantly slower turnover of these predominantly non-histone proteins than of other cellular proteins. Extensive ^{35}S-methionine labeling of rat pituitary tumor cell cultures (collaboration with Dr. N. L. Eberhardt, Department of Medicine, University of California, San Francisco) followed by isolation of poly ADP-ribosylated proteins by the affinity chromatography technique, then analysis in one dimension by electrofocusing, second dimension by electrophoresis (SDS-slab gel technique) disclosed the existence of at least 28 peptides that were isolated by this method, demonstrating covalent poly ADP-R binding. Actin and a group or predominantly non-histone proteins were identified and only a small amount of histone type proteins were found, confirming earlier results(1).

In vitro experiments with chromatin preparations isolated from normal and precancerous hamster livers. As an extension of results published in ref. 2, the total quantities of poly ADP-R, consisting of pre-existing endogenous poly ADP-R and newly formed ^{14}C-labeled chains synthesized in vitro by incubation with ^{14}C-NAD^+, were determined with the aid of the vitro chromatin assay system (see 2). Both radiochemical and chemical analytical methods were combined on the same incubation system.

*Despite the significantly larger incorporation of ^3H-thymidine into DNA in precancerous livers no significant increase in DNA content occurs, indicating also an increased rate of DNA breakdown. The question arises whether or not the increased ADP-ribosylation of the DNA-ase inhibitor actin (Fig. 2, Tables II, IV) counteracts the inhibitory effect of action on DNA-ase. This problem is being studied.

As shown in Table II pretreatment with the hepatocarcinogen for one month resulted in the large increase in endogenous poly ADP-R content (compare with Table I, Part 2) but only a small fraction of newly formed ADP-R oligomers were synthesized in vitro. Relatively larger quantities of newly formed oligomers synthesized in chromatin systems prepared from control animals containing ten times smaller amounts of pre-existing endogenous poly ADP-R.

These results illustrate that relatively limited information can be abstracted from customary radiochemical enzyme assays alone (see Table II), and without the quantitative approach developed in the course of these studies most of the significant observations would have remained undetected.

Two methods were used for the study of the nature of chromatin proteins that served as acceptors for newly formed ADP-R oligomers in the in vitro chromatin assay system.
First method: SDS-tartaramide-gel electrophoresis, followed by radioautography and gel scanning of radioautograms. This method provides an estimation of molecular weights of peptides that are templates for newly formed radioactive ADP-R oligomers. It does not determine the quantity of protein acceptors nor the total amounts of poly ADP-R, but indicates only the quantity of newly formed radioactive ADP-R oligomers. Since in exploratory experiments actin was tentatively identified as a specific non-histone protein ADP-R acceptor of chromatin, preparations from both normal and precancerous hamster livers were incubated ± actin and labeled NAD^+. Results are shown in Fig. 2.

It is apparent that in all chromatin systems a labeled protein with a molecular weight of 42 KD could be detected and addition of authentic actin (obtained as a gift from Dr. M. Morales, Univ. California, San Francisco) proportionally increased the ADP-ribose labeled protein which was indistinguishable from actin. ADP-ribosylated actin could be re-isolated and extracted from the gel system. Generally a significantly larger labeling occurred in peptides of chromatin that were isolated from precancerous hamster treated for one month with the hepatocarcinogen, consistent with results summarized in Table I, Part 2. A large molecular weight fraction was seen in the gel near the origin (fr. 1) suggesting the presence of long chain, probably cross linked poly ADP-R-protein complexes. Only trace amounts of histones could be identified (by mol. wt.), confirming earlier results (1) that non-histone proteins are indeed the predominant poly ADP-R acceptor proteins of chromatin. The radioactive bands in the gel were sliced, extracted and analyzed individually, as shown in Table III.

Histones and actin were re-isolated and their poly ADP-R content determined. Results are summarized in Table IV. It is apparent that actin added to a chromatin preparation that performs anzymatic ADP-ribosylation is about 10 times more ADP-ribosylated (on a molar basis) than the combined histone fraction of chromatin. This observation is again consistent with previous results (1) that identified non-histone proteins as primary ADP-R acceptors.

Identification of actin as a major ADP-R acceptor in chromatin predicts a new experimental approach to the study of the regulatory effect of ADP-ribosylations in chromatin. It is known that actin is the naturally occurring inhibitor of DNA-ase I (4); therefore if ADP-ribosylation modifies this inhibitory effect of actin then the NAD → poly ADP-R pathway represents a molecular mechanism that regulates nucleolytic activity during various biological conditions.

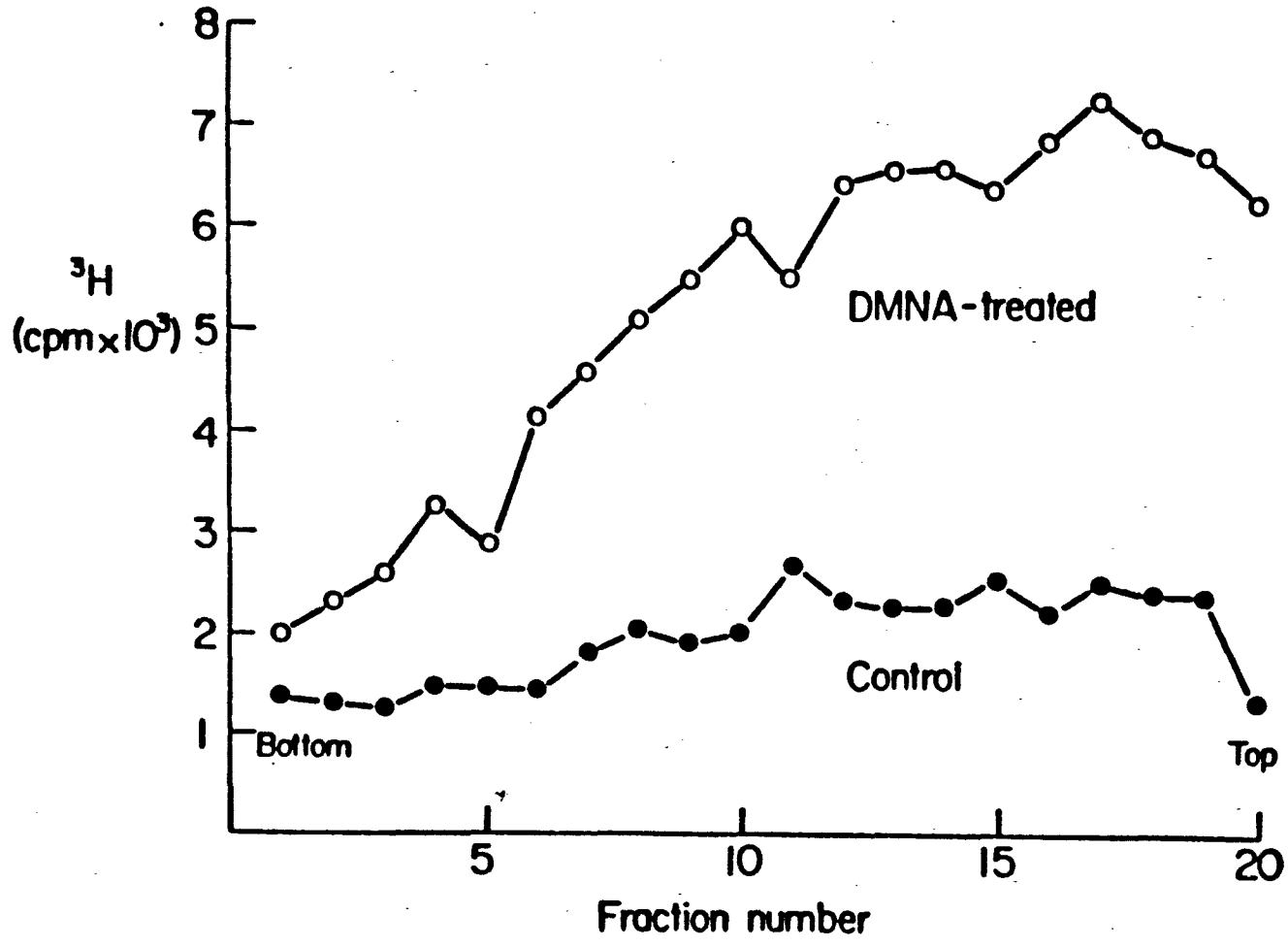


Fig. 1. Alkaline sucrose density gradient profile of DNA labeled in vivo.

Legend to Fig. 1.
Alkaline sucrose density gradient profile of DNA labeled in vivo.

Nuclei disrupted in 2% SDS + 20 mM EDTA equivalent to 0.22 mg DNA were layered on a linear sucrose gradient (5 to 20%) containing 100 mM NaCl, 100 mM NaOH. Ultracentrifugation at 300,000 x g for 1 h, followed by fractionation and radiochemical assay in fractions for DNA by the glass fiber filtration technique. The experiment was performed 18 hours after pulse labeling of DNA in vivo with 200 μCi ^{3}H-thymidine/100 g body weight.

Table II

Poly(ADP-R) content of normal and precancerous* hamster liver nuclei following *in vitro* incubation with 0.5 mM NAD^+ (fluorometry)

Nuclei	Poly(ADP-R) in nmoles/mg DNA		
	Total	Pre-existing	Synthesized
Control	33	20	13
Precancerous	211	200	11

*Treated for 1 month with DMNA 0.5 mg/week.

twelve mg DNA equivalent samples of chromatin were incubated for 30 min at 30° with 0.5 M NAD^+ (^{14}C-labeled in the adenine moiety) in 100 mM Tris-HCl buffer, 20 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 6 mM KF in a final volume of 2 ml. Pre-existing poly ADP-R was determined by the isolation of poly ADP-R-protein adducts by the boronate affinity chromatography method and subsequent fluorometric analysis of base dissociated poly ADP-R following hydrolysis (see scheme in V). Newly formed poly ADP-R chains were determined by radiochemical analyses.

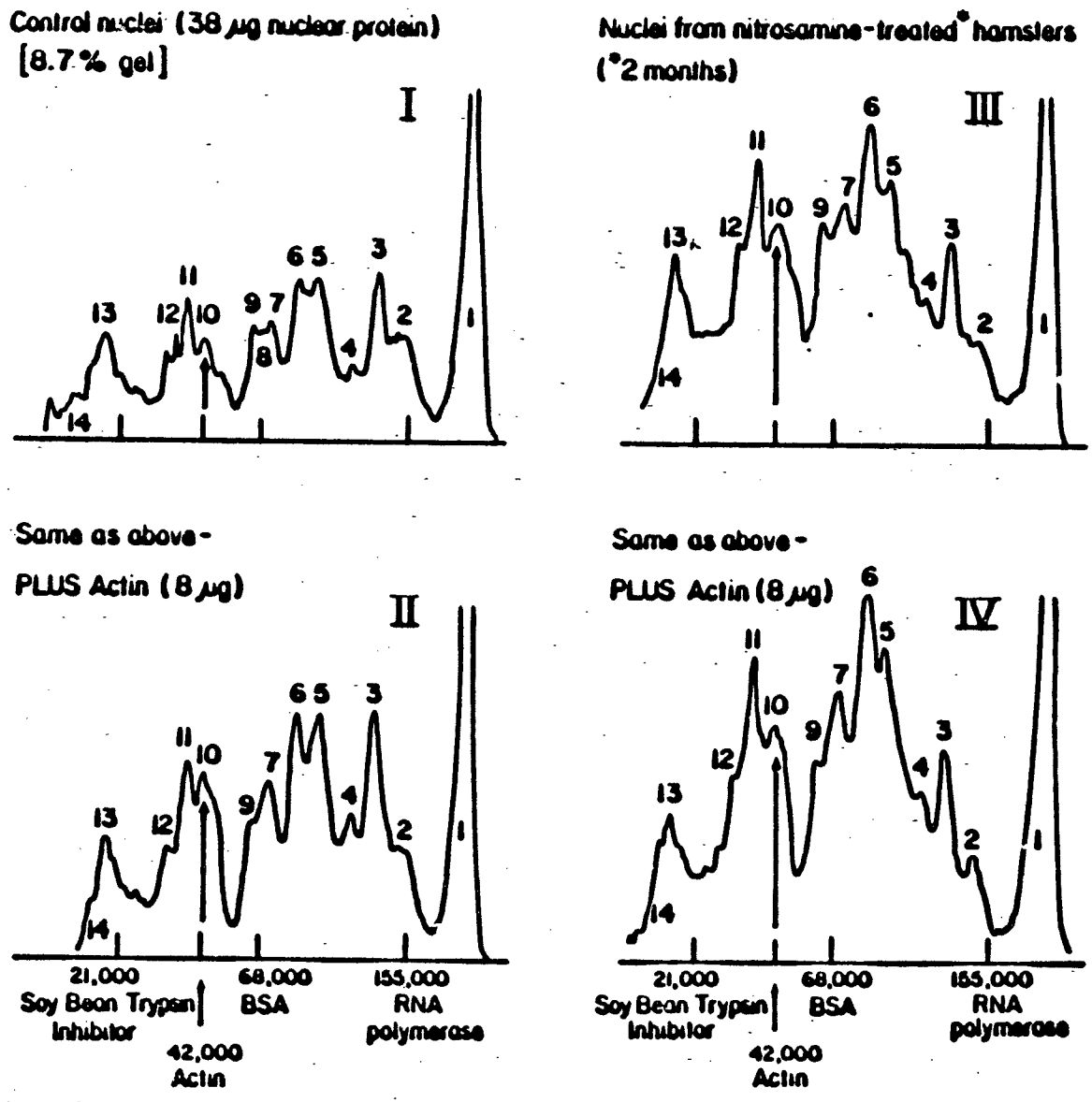


Fig. 2. Electrophoretic separation of poly ADP-R protein adducts

Legend to Fig. 2.

Title: Electrophoretic separation of poly ADP-R protein adducts.

Incubation conditions were the same as described in the legend of Table I, except on a small scale (38 μg nuclear proteins/incubate). The TCA (10%) precipitated protein adducts were washed 4 times with 10% TCA, then TCA was removed by diethylether, and the precipitate dissolved in a buffer containing 1% SDS, 15% sucrose, 10 mM Tris (base), 10 mM NaCl, 1 mM EDTA, 40 mM DTT pH 7.5. The gel electrophoresis was run on 8.7% N,N'-diallyltartardiamide cross-linked polyacrylamide gel (soluble in 2% HIO_4). Coomassie blue staining and radioautography were performed on separate but identical gels.

Ordinate: relative absorbance as recorded on gel scanning apparatus; gel scan of autoradiograms.

Abscissa: electrophoretic mobility (from right to left) and mol. wt. standards.

Table III

^{14}C-ADP-R content of gel fractions shown in Fig. 2

Molecular Mass	Fraction Number	Control	Control + actin	DMNA treated	DMNA treated + actin
>155KD	1	18,163	18,427	18,449	21,566
155KD	2	887	1,418	1,796	1,386
	3	1,818	1,972	2,280	2,491*
	4	880	1,625	1,855	2,237*
	5	2,458	2,835	3,035	4,977*
	6	3,501	3,090	4,505*	6,084*
60KD	7	1,918	2,080	2,572	4,398*
	9	1,320	2,122	2,062	2,318
42KD actin	10	2,093	3,470	3,802	5,511*
	11	2,001	2,264	3,039*	3,452*
	12	1,471	1,948	2,478*	2,604*
20KD histones	13	1,555	1,499	2,850*	2,080*
20KD histones	14	1,617	878	1,854	1,746

Results: counts (^{14}C) per 5 min.

Note: fraction 8 was included into slice 9.

The gels were sliced and after dissolution in 2% HIO_4 ^{14}C content determined by scintillation spectrometry.

Table IV

In vitro
Poly(ADP-ribosylation) of endogenous histones and of added actin by chromatin of hamster liver

No	Preparation	m moles ADP-R per mole		Histone content
		Actin	Histone	µg/ test
1	Control + 6.4µg actin	60.1	5.6	17.9
2	Control (no additions)	-	7.5	17.0
3	DMNA treated + 7.1µg actin	85.9	9.1	18.1
4	DMNA treated (no additions)	-	11.1	17.7

Second method: The second experimental approach for the study of the nature of in vitro poly ADP-ribosylation was HPLC-molecular filtration of ADP-ribosylated proteins that were isolated by the boronate affinity chromatography method. Both radioactivity (ADP-R moiety) and absorbance at 280 nm were monitored. It would be expected that only relatively large amounts of proteins would be detectable by UV, whereas small quantities of ADP-ribosylated proteins would be readily determined by ^{14}C-analysis. As shown in Fig. 3 in chromatin isolated from both normal and precancerous livers a large molecular weight protein fraction (larger than 158 KD) was separated (assayed both by ^{14}C counts and UV) by molecular filtration. This protein fraction seems to be identical with fr. 1 in Fig. 1. However, in the poly ADP-R-protein complex isolated from precancerous livers (two month treatment with the hepatocarcinogen) a significant, UV detectable ADP-ribosylated protein of about 47 KD was detectable. The nature of this protein fraction is being further studied, and present information does not exclude the possibility that the molecular filtration method may signal a higher app. mol. wt. for actin as compared to SDS gel electrophoresis; thus the protein fraction could be ADP-ribosylated actin.** The total quantity of newly formed radioactive ADP-R oligomers is about 50% higher in the protein complex isolated from in vitro ADP-ribosylating chromatin or precancerous livers.

** The mol. wt. of G-actin has been reported earlier as 55 to 66 KD contaminated with "heterodisperse aggregates". Later, mol. wt. was stated to be between 42 and 48 KD (5). Variation in app. mol. wt. may reflect different degrees of poly ADP-ribosylation of either actin only or in addition to actin poly ADP ribosylation of actin associated (regulatory?) proteins. Presently DNA-ase Sepharose affinity column in applied for actin studies.

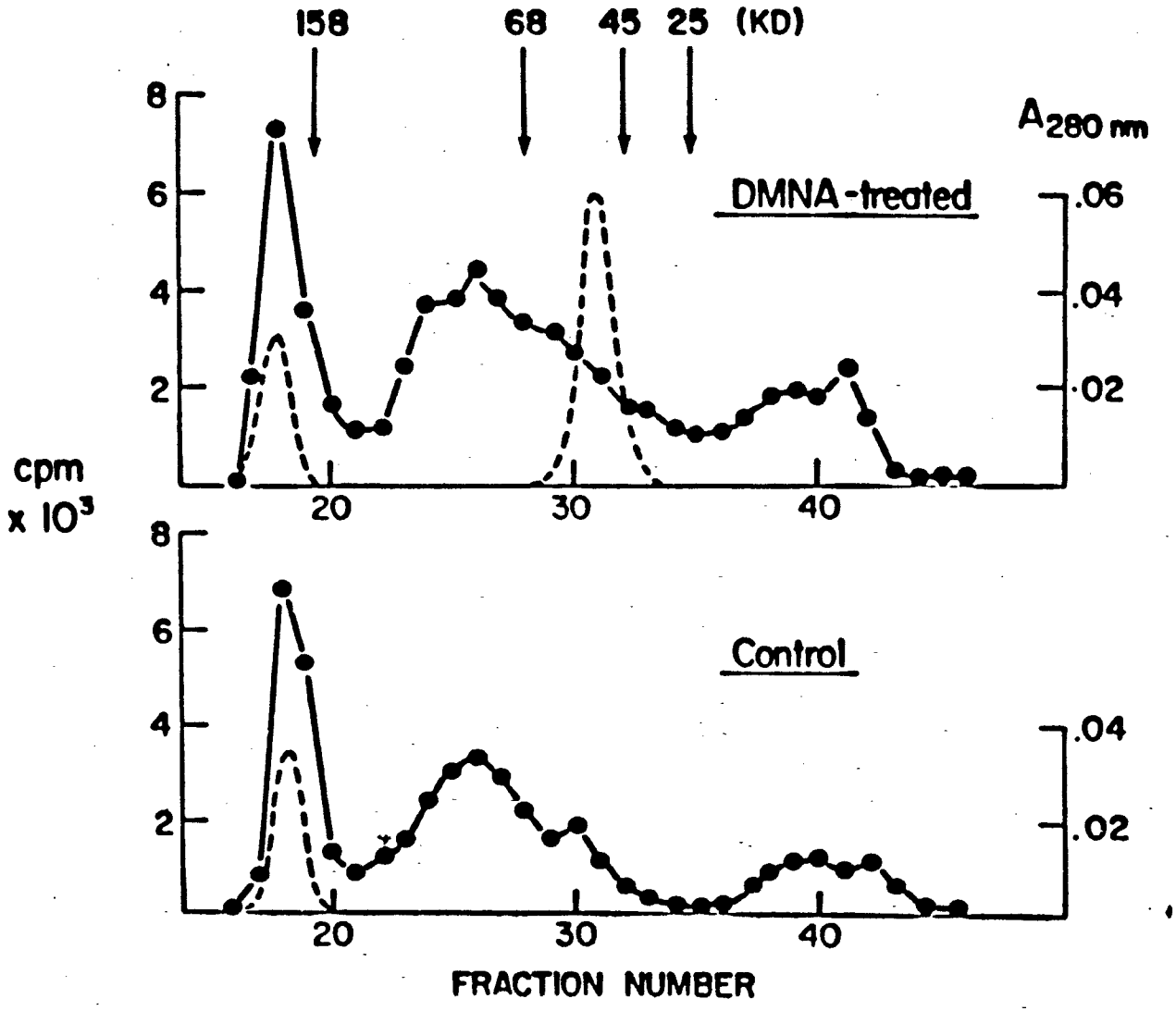


Fig. 3. Molecular Weight profile of in vitro poly ADP-ribosylated proteins isolated by boronate affinity chromatography (HPLC, column TSK-3000 SW mol. sieve).

Legend to Fig. 3.

Title: Molecular weight profile of in vitro poly ADP-ribosylated proteins isolated by boronate affinity chromatography (HPLC, column TSK-3000 SW mol. sieve).

The quantity of poly ADP-ribosylated protein is calculated on the basis of 0.22 mg DNA present in both incubation systems. incubation systems were the same as described in the legend of Table II. This was injected into the HPLC system in a volume of 200 µl and developed with 1.5 M guanidine carbonate containing 0.1 M acetate buffer (pH 6.0). Fraction vols. = 0.5 ml; flow rate = 0.4 ml/min at room temperature. UV absorbance at 280 nm was simultaneously monitored in the HPLC apparatus and radioactivity determined in each 0.5 ml fraction.

NEWLY DEVELOPED METHODS AND EXPERIMENTAL DESIGN.

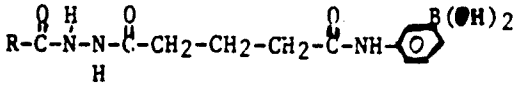
The following new methods have been developed and are being adapted for research aimed at solving the problems listed in objectives.

Methods

1. Dihydroxyboryl affinity chromatography

Purpose: isolation of ADP-R-protein adducts and of ADP-R oligomers after alkaline cleavage of ADP-R-protein bonds. This affinity chromatography, originally introduced by Inman and Dintzis (6), was adapted to the synthesis of highly substituted acrylamide type molecular sieve base extended with a linker. A high degree of specificity was achieved that is much superior to sepharose-base affinity columns, the latter exhibiting only 50-70% efficiency in selectively absorbing ADP-R oligomers, whereas the poly acrylamide-glutaryl-hydrazide-m-aminophenyl boronic acid column binds reversibly at least 90% of poly ADP-R proteins.

Synthesis of m-aminophenyl-boronic acid glutaryl hydrazide poly acrylamide affinity column.



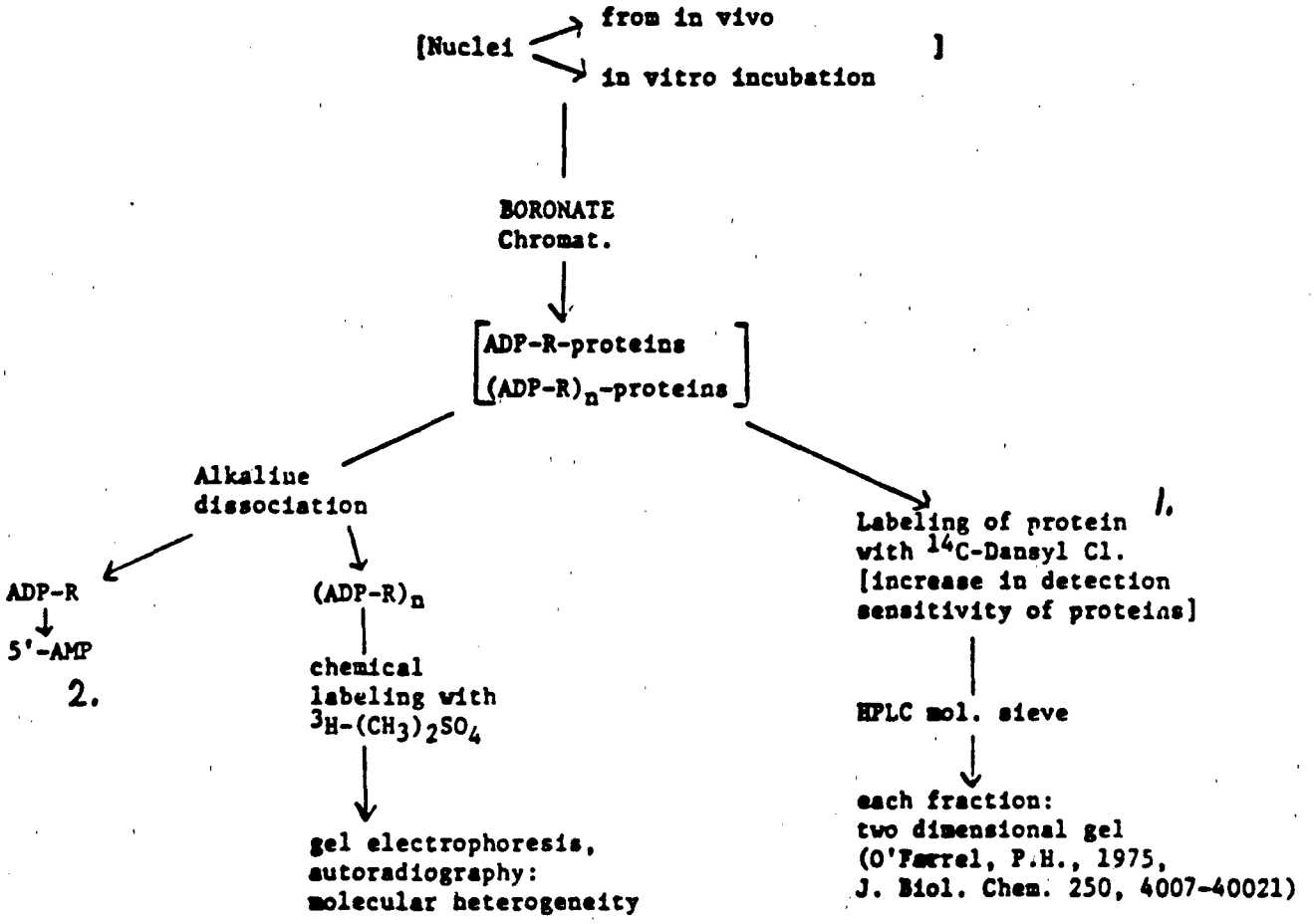
Initial steps were adapted from ref. 6 and Methods in Enzymology (Vol. XXXIV, 30-58, 1974).

Ten g of Biogel P-300 (50-100 mesh) were stirred for 5 hours at 50° in 6N hydrazine hydrate (600 ml) and allowed to stand for 1 h to cool, then extinsively washed with 0.2 N NaCl in a coarse sintered glass funnel until traces of hydrazine were no more detectable in the eluate by the trinitrobenzene-sulfonic acid color test. The hydrazine gel was further washed with 10 vol. of 0.1 N NaCl and resuspended in the same solution. Recrystallized glutaric anhydride was added: 3 mmoles/moles hydrazine (on resin). This was 2.5 to 2.9 m equ./g dry wt. resin. The pH was kept at 4.0 during the entire procedure, which lasted 1 h (by small additions of 2 N KOH). The carboxyl content was at the end 2.5 - 2.9 m equ. per g dry wt. of resin. The final condensation of m-aminophenyl boronic acid was catalyzed by EDAC (1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide HCl), added as 1.5 molar equiv. (to m-amino phenyl boronic acid) at pH 5.5 for 16 h at 4° and 1.5 fold excess (to hydrazine resin) m-aminophenyl-boronic acid. The extent of reaction was followed according to ref. 7 , and this was found to be nearly quantitative under these conditions. The resin was resuspended in 6 M guanidine·HCl (or carbonate)-NaAc 100 mM, pH 4.0 and washed free from m-aminophenyl-boronic acid. Absorption of poly ADP-R-proteins (at pH 8.2), removal of DNA, RNA and proteins and desorption of ADP-R-proteins at pH 6.0 is illustrated in Fig. 5 of ref. 2 . The nucleic acid specificity of the column was tested. Since it seemed possible that traces of RNA could be absorbed due to ribose at RNA termini, commercial yeast RNA and DNA were methylated (at the adenine or deoxyadenine sites) with ^{3}H dimethylsulfate (8) and the tritiated RNA and DNA passed through the column. DNA did not absorb to the column but a trace (less than 0.4%) of RNA was absorbed. From comparison of ^{14}C-poly ADP-R and ^{3}H-RNA

adsorption tests it was apparent that a maximum of 7 to 10% error (interference) by RNA can occur, but this error falls within the limits of accuracy of the technique for the analysis of poly ADP-R-protein adducts (± 15-20% S.D.), and therefore does not measurably falsify results. Furthermore, RNA completely dissociates from protein in 6 M guanidine HCl and the only proteins that are separable by the boronate affinity column (cf. 2) are proteins containing covalently bound ADP-R; therefore the method is specific for these proteins. This was also tested by first dissociating poly ADP-R from proteins at pH 10.5, then passing the mixture through the boronate column. No measurable retention of proteins, only of ADP-R, was observed (cf. 2), indicating also that no interference by glycoproteins can be detected in chromatin extracts. Chromatography at pH 8.2 does not measurably dissociate ADP-R-protein bonds; they cleave at pH 10.5.

2. Isolation of protein (ADP-R)n adducts from nuclei. Poly ADP-R was found to be unexpectedly stable in nuclei isolated by conventional techniques either in sucrose or in citric acid (cf. 9), provided a protease inhibitor was present during isolation. Poly ADP-R degrading enzymes (as well as enzymes involved in NAD^+ synthesis) are readily extracted during isolation of nuclei during homogenization. For this reason it was not necessary to continue with freeze clamping (cf. 1) of total tissue samples and analyses of isolated nuclei gave representative values found by the freeze clamp technique (1). Either after in vivo labeling (^{14}C-ribose) or after in vitro incubation of sonicated nuclei (chromatin) with labeled NAD^+, proteins were precipitated and washed 5 times with 500 vol. of 10% TCA (4°), quantitatively removing soluble nucleotides. The precipitate was dissolved in 6 M guanidine HCl (or carbonate) adjusted to pH 8.2 with 100 mM morpholine buffer, quantitatively (more than 99%) solubilizing all radioactive material, and the solution centrifuged at 27,000 x g for 20 min. to remove fibres. Adsorption of protein ADP-R adducts at pH 8.2 is shown in ref. 23. Column size was 0.5 to 1 g boronate resin, depending on sample size. After loading (15-20 min) and wash (160-170 ml 6 M guanidine HCl, -morpholine, pH 8.2) the resin material was quantitatively removed from the column and stirred in a beaker with 20 - 30 ml of 100 mM NaAc., pH 4.0 (pH was titrated to this end point with 2 N HCl), the column was repacked and elution continued at pH 4.0. Eighty-five to 90% of poly ADP-R-protein adducts were recovered. The eluate was concentrated on Amicon YME filter to 1-2 ml. Depending on the purpose of further steps, the solution was either analyzed on HPLC-mol. sieve (Fig. 3) or by gel techniques (Fig. 2), or cleaved to free ADP-R oligomers at pH 10.5 for 6 hours(20°). Proteins were precipitated by $HClO_4$ (10%), ClO_4^- removed as K^+ salt and poly ADP-R analyzed either by immunochemical or chemical methods, by enzymatic degradation to characteristic nucleotides (10) followed by HPLC separation on an anion exchange column either as free nucleotides or derivatized by chloroacetaldehyde as fluorescent products (7), detected fluorometrically. When non-labeled poly ADP-R was isolated by the above techniques tritiation of oligo and poly ADP-R (and of ADP-R) by ^{3}H-dimethyl sulfate and subsequent gel electrophoresis (11) provides an ultramicro method for chain length analyses since the gel technique separates oligomers differing only one ADP-R unit. Combination of the boronate technique with this gel method is presently substituted for the more laborious enzymatic methods for the molecular size analyses of poly ADP-R. The tritiation by ^{3}H-dimethylsulfate of poly ADP-R was developed following a published method for DNA and RNA (12).

SCHEME OF COMBINED ANALYTICAL METHODOLOGY



1. When ADP-R is labeled, first dissociate from protein is base, then label protein with ^{14}C-Dansyl Cl.

2. RNA yields 2' and 3'AMP, which are readily separated from 5'AMP by HPLC on anion resin with PO_4 gradient. 2' and 3'AMP do <u>not</u> bind to the boronate column and thus do <u>not</u> interfere if at <u>2</u> a second boronate column is introduced.

The above scheme provides a complete analysis of all species (mono, oligo ADP-R proteins) and also includes independent internal controls (e.g. comparison of immunoassay with (ADP-R)n analyses by chemical methods). Soluble nucleotides (e.g. NAD^+) are determined by HPLC separation, UV monitoring and—if labeled—by radiochemical methods.

3 Separation of nuclear populations by subfractionation of a mixed population. This method is being used to obtain nuclear fractions from various cell types of liver cells.

Rationale of the chosen method. Two alternative experimental avenues exist for the separation of cell nuclei derived from various cell types that comprise an organ. Cells can be initially separated by well known perfusion methods (with collagenase + hyalorunidase) capable of disrupting cellular interactions. This investigator's experience indicates that metabolically functional hepatocytes can be separated by the above perfusion methods, but chromatin of collagenase treated liver cells is often damaged, and poly (ADP-R) content always decays during perfusion of livers with collagenase. For this reason we first isolate an intact mixed nuclear population from the whole organ and subsequently differentiate nuclear subpopulations by an isopycnic centrifugation method. It should be noted that zonal centrifugation has been successful in separating "precancerous" nuclei from thioacetamide treated rat livers (12).

Centrifugal method.*

a) One g of liver is homogenized in 5 volumes of medium (0.25 M sucrose, 10 mM Tris-Hepes pH 8.0, 3 mM $MgCl_2$, 0.1 mM PMFS) and total nuclei separated by sedimentation at 1000 x g for 10 min at 4°.

b) The crude nuclear pellet (from a) is resuspended (homogenized) in 5 vol. of medium and fibers are removed by filtration through 4 layers of nylon cloth; the nuclei are resedimented (1000 x g for 10 min at 4°).

c) The sediment obtained in b) is rehomogenized in 5 vol. of medium, now containing in addition to previously described components also 0.1% Triton X-100, and nuclei are resedimented as in b).

d) The Triton-treated nuclear pellet of c) is rehomogenized in 5 vol. of 2.2 M sucrose containing 10 mM Tris-Hepes pH 8.0, 3 mM $MgCl_2$, 0.1 mM PMFS and centrifuged in a preparative ultracentrifuge (model L5-50B in SW-28 rotor at 113,000 x g for 60 min at 2°.

e) The purified nuclei obtained in d) are resuspended in 2.0 M sucrose, 10 mM Tris-Hepes pH 8.0, 7 mM $MgCl_2$, 0.1 M PMFS and layered on a discontinuous sucrose gradient (in centrifuge cups of the SW-28 system). The total volume is 17 ml, discontinuous sucrose layers are 3 ml each varying from top 2.3 M to bottom 2.85 M. Separation is achieved in the ultracentrifuge at 118,000 x g for 2 hours at 2°.

The results of such an experiment are shown in Fig. 4. The subpopulation of nuclei are separated by gradient-slicing, and nuclei are being characterized by microscopic examination, DNA, RNA and poly(ADP-R) polymerase assay, as well as by chemical analyses (protein, DNA, RNA and poly(ADP-R)-protein adducts).

Assay for poly ADP-R-glycohydrolase in tissue samples. Adaptation of the HPLC separation of ADP-R on an anion exchange (HPLC) column by PO_4 gradient provides a specific micro assay for poly ADP-R-glycohydrolase. The method is suitable for enzyme assay in homogenates (in 0.15 M KCl-Tris, pH 7.4). Sonication of homogenates

*Adapted from Jackowski, G. and Liew, CC., Biochem. J., in press. Dr. Jackowski is joining our laboratory in April 1980.

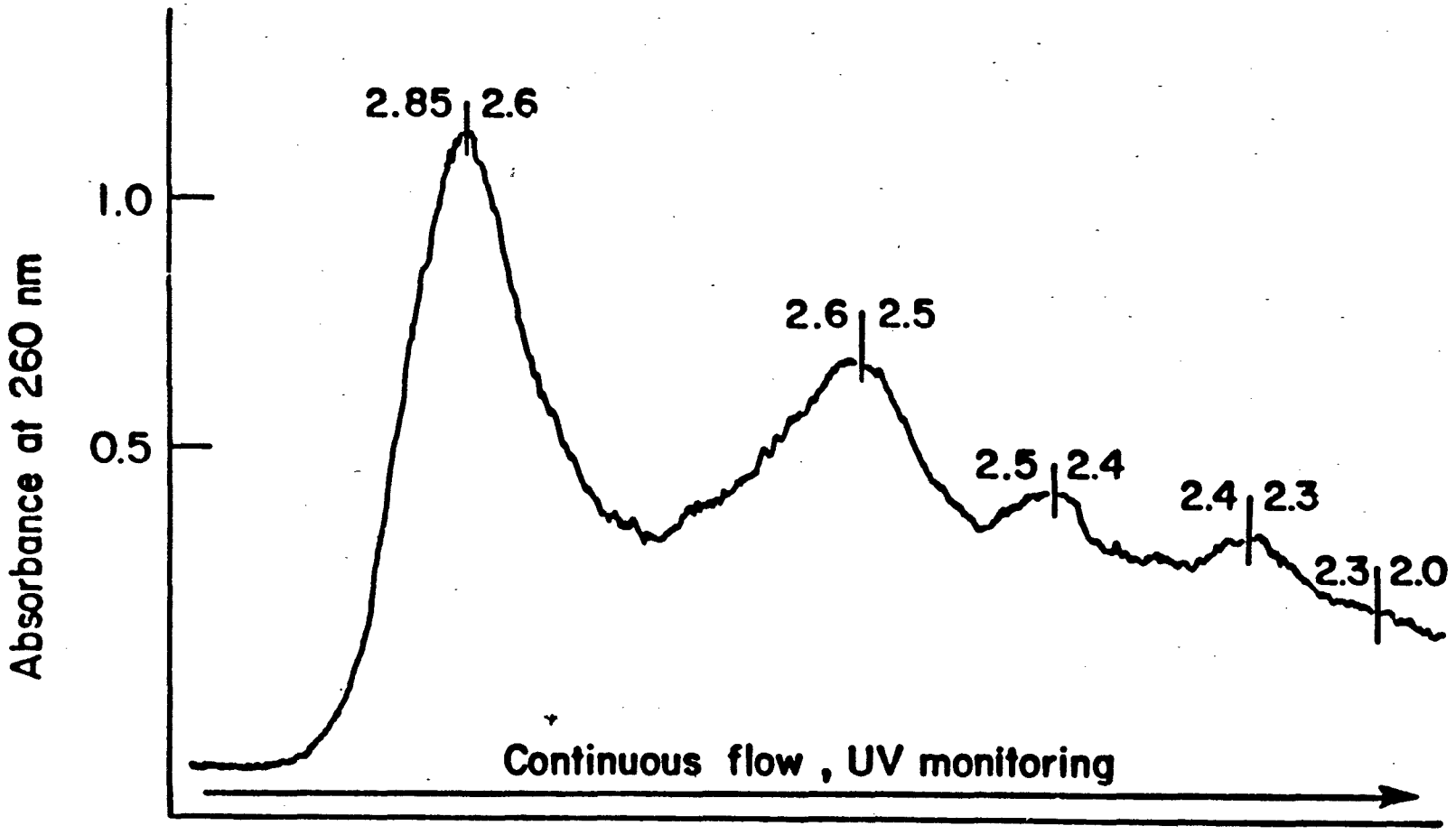


Fig. 4. Separation of nuclear subfractions by discontinuous sucrose density gradient ultracentrifugation. [Numbers indicate density of sucrose.]

and sedimentation of particles quantitatively extracts the glycohydrolase from nuclei (13). Incubation of poly ADP-R (n = 30) with the soluble extract, followed by deproteinization ($HClO_4$) and HPLC analysis of the extract for ADP-R released is a direct assay for the enzyme. Optimal assay conditions are presently being developed for the assay of this enzyme in animal tissues.

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PART 2. RESULTS OBTAINED AFTER FEBRUARY 1980 UNTIL OCTOBER 1980

In contrast to a large variety of covalent modifications of both histone and non-histone proteins by phosphorylation, acetylation, methylations etc., reactions that appear to exhibit a relatively broad selectivity, poly ADP-ribosylation by $(ADP\text{-}R)_n$ in small rodents *in vivo* is confined to an extent of at least 99% to non-histone chromosomal proteins, as determined by an $(ADP\text{-}R)_n > 4$ specific IgG antiserum assay (Minaga et al., 1979). Similar results were recorded in another laboratory in varying biological material by different experimental methods (Bradehorst et al., 1978; Bradehorst et al., 1979). It should be noted that ADP-ribosylation of histone H_1 *in vitro* is readily demonstrable when larger quantities of the ADP-R acceptor histone H_1 are added to the purified poly ADP-R synthetase (Kawaichi et al., 1980), but this is a typical *in vitro* enzymological result. Quantitatively misleading results can be obtained *in vivo* or, in cell cultures, when only radiochemical assays of ADP-R are the basis of analyses without consideration of the absolute quantities of $(ADP\text{-}R)_n$ (Giri et al., 1978; Jump et al., 1979). It is obvious that measurement of radioactivity alone does not distinguish between small quantities of highly radioactive $(ADP\text{-}R)_n$ or larger amounts of $(ADP\text{-}R)_n$ with lower specific activty, both parameters being related to turnover (Kun et al., 1979). There is no doubt that only about 0.1 to 0.6% of the total $(ADP\text{-}R)_n > 4$ is associated *in vivo* with histones and shorter as well as longer homopolymers

exist as covalent complexes with histones (Minaga et al, 1979). However, the overwhelming targets of ADP-ribosylation are non-histone proteins that have been identified by the pioneering experiments of Paul and Gilmour (1968) to play an as yet not well understood role in the regulation of differentiated cellular functions in eukaryotes. The previously proposed correlation between transcriptionally active chromatin and the degree of ADP-ribosylation (Mullins et al., 1977) is presently doubtful, since more recently no connection was found between the localization or activity of poly ADP-R polymerase and transcriptional activity of chromatin (Yukioka et al., 1978).

Since positive correlation between a variety of differentiated cell functions as well as differentiation itself and the regulatory role of non-histone proteins is probable (Paul and Gilmour, 1968) we pursued the problem of the regulatory function of poly ADP-ribosylation in experimental models that express the operation of development and differentiation: early phase of carcinogenesis, age dependent and hormone influenced organ-specific nuclear responses. The present paper is concerned with on-going experimental work in this area. Results are consistent with the working hypothesis that predicts a major cellular regulatory function of poly ADP-ribosylation by way of modification of non-histone proteins. This modification process can be influenced either at the transcriptional level resulting in variations of the synthesis of enzymatic components of

the poly ADP-R synthetase systems or by changes in the concentration of target non-histone proteins, or both. A unique metabolic effect on chromatin function through this system is suggested by the variation of ADP-ribosylations due to metabolically induced changes in NAD^+/NADH ratios (Kun and Chang , 1976) possibly contributing to the initiation signal of stress-induced organ hypertrophy (Zak and Rabinowitz, 1979).

2. Evaluation of methodology for in vivo studies

The advantage of in vivo experimentation is its obvious relevance to physiologic function. Physiologic interpretation of in vitro enzymology in a relatively undeveloped field requires constant re-evaluation of in vitro systems, since it is uncertain whether or not the chosen in vitro system contains all regulatory components present in the intact cell. This plurality of experimental approach is reflected in experimental methods, developed in our laboratory. Notable advances in related areas were made also in other laboratories, but the present paper will be confined to the analysis of techniques used in our research group.

2.1 Immunochemistry of poly(ADP-R)n > 4

The quantitative analysis of the homopolymer poly(ADP-R) by radiochemistry is hampered by the absence of a specific precursor. Labelled ribose, or ^{32}P are suitable and ^{14}C-ribose is preferable

because of its convenience of handling. Adenine is an unsuitable precursor *in vivo*, probably because preferential pathways other than those leading to poly(ADP-R) obscure the labelling of the homopolymer. Development of a highly specific anti-poly(ADP-R) IgG rabbit globulin that reacts with (ADP-R) > 4 (Ferro et al., 1978) significantly aided our experiments. An improved extraction procedure was also developed that was used to determine the distribution of the polymer among various types of nuclear proteins (Minaga et al.,1979). Combination of immunochemical analyses and *in vivo* labelling (Minaga et al., 1979; Kun et al., 1979) gave a $t_{\frac{1}{2}}$ for NAD^+ and of poly (ADP-R) of 2.7 and 3.1 h respectively, in terms of ribose incorporation, the slight discrepancey representing experimental variation only.

2.2 Microchemical Methods

The disadvantage of the immunochemical technique is twofold. First, the preparation of the antibody may be variable and second, the method does not detect oligomers smaller than tetramers. For these reasons entirely chemical micro procedures were developed. Poly and mono-ADP-ribosylated nuclear proteins were quantitatively isolated on a highly substituted affinity column: m-aminophenyl-boronic acid glutaryl-hydrazide poly acrylamide (Romaschin et al., 1980), that exhibits better than 90% recovery of ADP-R-proteins, in contrast to 50-70% of polysaccharide based columns containing

no spacers. The isolated ADP-ribosylated nuclear proteins, containing in vivo administered labelled precursors (either ^{14}C-ribose for ADP-R, or labelled amino acids as protein markers) can be directly analyzed by HPLC-molecular sieve columns (Romaschin et al., 1980) or hydrolyzed to free $(ADP\text{-}R)_n$ and proteins. In case the protein was not labelled, ^{3}H-dansylation is performed and the macromolecular protein profile of the ADP-R free target proteins of ADP-ribosylation are subjected to HPLC-molecular filtration, combined with isotope analyses (Romaschin et al., 1980). For more detailed peptide separations the proteins are labelled with ^{125}I and subjected to one-dimensional electrofocusing and SDS-electrophoresis in the second dimension. Protein-free ADP-R oligomers are degraded by purified poly(ADP-R) glycohydrolase, and ADP-R analyzed as a fluorescent derivative on an anion exchange column by HPLC-fluorometry, using a phosphate gradient and combined with isotope dilution with ^{14}C-ADP-R. Less than picomole quantities are readily measurable. Alternative methods were developed for the determination of macromolecular profile of ADP-R oligomers. Either HPLC-molecular filtration (when the homopolymer contains ^{14}C-ribose) was performed to determine the newly formed oligomers, detected by ribose labelling (representing mostly chain elongations and internal randomization of ^{14}C-ribose with the unlabelled ribose pool) or by ^{3}H-dimethylsulfate labelling of all $(ADP\text{-}R)_n$ species, followed by gel eletrophoresis, radioautography and densitometry. A combination of above techniques

is employed in our laboratory for the quantitative assay and macromolecular association of both monomeric and oligomeric $(ADP\text{-}R)_n$-protein adducts. Good agreement between immunochemical and chemical analyses was obtained (Minaga et al., 1979; Kun et al., 1979; Romaschin et al., 1980). It was also confirmed that in normal animal tissues there are no free ADP-R oligomers unless artificially produced by the action of intranuclear proteases during in vitro manipulations.

3. Quantitative studies in vivo during the early precancerous state induced by dimethyl-nitrosamine treatment of Syrian hamsters.

The first model related to pathophysiological alterations of differentiation in animals was the early stage of hepatocarcinogenesis in Syrian hamsters treated with small doses of dimethylnitrosamine (weekly i.p. injection of 0.5 mg per 100 g body weight for one or two months, Harrold, 1956). As described in the paper by Romaschin et al. (1980) treatment of one-month-old hamsters for one month (four injections of 0.5 mg dimethylnitrosamine/100 g) resulted in a five-fold increase in chemically determined poly(ADP-R) and a significant increase in the incorporation of in vivo administered ^{14}C-ribose (50μCi/100 g) into both poly(ADP-R) and NAD^+, indicating an increased turnover of the pathway $NAD^+ \rightarrow$ poly(ADP-R). There is simultaneously a decrease in the steady state concentration of NAD^+ ($\sim$ 25%), consistent with its increased utilization for the protein-

ADP-ribosylating pathway. Continued treatment of hamsters for two months diminished the difference between steady state concentrations of poly(ADP-R) between normal and hepatocarcinogen-treated hamsters, but further increased the rate of flux between NAD^+ and poly(ADP-R). Notably the turnover of ribose in two-months-old animals diminished, indicating an age dependent variation of NAD^+ and poly(ADP-R) metabolism, however the carcinogen induced increase is independent from this age-related change and is only dependent on the dose and time of administration of the carcinogen. These results are summarized in Table I. Separation of labelled poly ADP-ribosylated proteins, prepared from one month precancerous hamster livers by CsCl density ultracentrifugation is shown in Fig. 1. It is apparent that the three macromolecular components, DNA, RNA and poly(ADP-R)-protein adducts are readily separable by this physical procedure. Separation and determination of the macromolecular profile of in vivo ^{14}C-ribose labelled protein-poly(ADP-R) adducts in 1.5 M guanidine formate solution by HPLC is illustrated in Fig. 2. Both normal and precancerous (one month treated) hamster livers were analyzed and the molecular profile (on HPLC) was monitored in a TSK-3000 molecular sieve. The molecular masses (in kilodaltons: kd) are plotted against fraction numbers. In these experiments no definite determination of the absolute quantities of poly(ADP-R) is possible because pulse labelling in vivo randomly labels elongating homopolymers and is also recycling with endogenous poly(ADP-R).

Quantitative asessment is always based on direct chemical analyses (see Table I). The protein-poly(ADP-R) adducts were hydrolyzed, the polymer-free proteins labelled with ^{3}H-dansylchloride and both proteins and homopolymers subjected to HPLC-molecular mass analyses. The homopolymers were separated on a TSK-2000 molecular sieve. Results are shown in Fig. 3. It is evident that in pre-cancerous liver nuclei poly ADP-ribosylation of proteins of a mass about 160 kd is greatly increased. The analysis of the molecular profile of free homopolymers indicated that only about 10 to 15% of <u>in vivo</u> mono ADP-ribosylation takes place and at least 80 to 90% of the homopolymer is larger than monomeric. These results extend and support previous analyses obtained by the $(ADP\text{-}R)_n > 4$ specific antiserum (Minaga et al., 1979) but in contrast to the immunochemical method, that does not assay smaller than tetramers, the chemical procedures reveals a detectable quantity of mono-ADP-ribosylation. However, the mono-mers are still only 10 to 15% of the oligomers.

<u>In vitro</u> pulse labelling of both normal and precancerous hamsters with ^{3}H-thymidine and analysis of incorporation into DNA revealed that in the precancerous animals the rate of thymidine incorporation increased about fourfold. Alkaline sucrose density gradient analysis of DNA species indicated an increase of DNA species of short chain length, but no typical fragmentation, as observed in tissue-cultures treated with high concentrations of

of alkylating agent (2 mM dimethylsulfate, cf. Durkacz et al., 1980), therefore at this early precancerous stage, where no morphologic evidence of malignancy is detectable, there is no evidence suggesting a correlation between DNA fragmentation and an increase of poly(ADP-R) metabolism. It is probable that 2 mM alkylating drug in vitro in tissue-cultures is a highly toxic dose, and DNA fragmentation reflects non-specific toxicity. It is known that the subtle transformation process to malignancy does not necessarily involve gross chromosomal alterations (Dipaolo, 1977) but coincides with much less obvious modifications of DNA. Our model, that focuses on an early stage of malignant transformation is consistent with this view (Dipaolo, 1977) therefore the large increase in poly(ADP-R) turnover and non-histone-protein modification precedes any gross alteration (fragmentation) of DNA, and is probably a very early signal of the precancerous state. This mechanism and its relationship to DNA-repair is an open question.

4. Rates of Poly(ADP-R) synthesis in cardiocyte nuclei as a function of age and organ-specific hormonal influences in vivo on rates of poly(ADP-R) Synthesis in isolated cardiocyte nuclei, in vitro effect of aldosterone.

We have previously shown that poly(ADP-R) synthesis in cardiac nuclei is two to three times higher than in livers (Ferro and Kun, 1976) of the same animals, suggesting a possible physiologically prominent function of ADP-ribosylation in cardiocytes. With the aid of a specific separation technique cardiocyte nuclei were isolated from a mixed nuclear population (Jackowski and Liew, 1980) and the effect of aging - an expression of development - on rates of in vitro poly(ADP-R) synthesis was determined. Striking results were obtained, as shown in Fig. 6. Rates of poly(ADP-R) synthesis in vitro were much higher in myocardial nuclei isolated from 30-day-old than from 65-day-old rats (Long Evans strain). These results are in apparent variance with an earlier report (Claycomb, 1975) that claimed higher poly-(ADP-R) synthesis in nuclei of older rats. Unfortunately Claycomb (1976) did not separate myocardial nuclei from nuclei of non-myocytes - as done in the present work (Jackowski and Liew, 1980) - and it is likely that the fortuitous relative enrichment of non-myocyte nuclei containing higher enzymatic activity than cardiocyte nuclei signalled a higher apparent total enzymatic activity. The possibility of this type of artifact has been actually considered by Claycomb (1976). In general, when rates of poly(ADP-R) synthesis are increased the steady state

concentration of nuclear NAD^+ decreases. Claycomb (1975) observed a decrease in NAD^+ content of cardiac tissue of young animals, an observation consistent with our results. The kinetic data shown in Fig. 6 support the view that aging markedly influences the poly ADP-ribosylation pathway.

In further studies we tested the apparent effects of endocrines on nuclear poly(ADP-R) synthetase activity. The most striking phenomenon observed was organ specificity. Only myocardial nuclei exhibited a hormone sensitivity, whereas liver nuclei were unresponsive. As illustrated in Fig. 7 pharmacologic dose of cortisol (5 mg/rat) severely depressed the poly(ADP-R) synthetase activity of myocardial nuclei, but not of liver nuclei. The relatively large effective dose of cortisol made it suspect that possibly a side effect of this hormone (mineralocorticoid-like action) was observed. The enzymatic activity in myocardial nuclei of hypophysectomized rats was markedly diminished - as would be anticipated - and cortisol at 5 mg/100 g dose and aldosterone at 5 µg/100 g dose further depressed activity, indicating that indeed, aldosterone was the more powerful inhibitory agent in vivo, and the large dose of cortisol mimicked the effect of the typical mineralocorticoid. These in vivo effects are shown in Fig. 8. Adrenalectomy significantly diminished poly(ADP-R) synthetase activity of myocardial nuclei and cortisol (5 mg/100 g rat) further depressed activity (Fig. 9). The effects of pituitary and adrenal hormones are clearly complex. Presence of both

pituitary and adrenal glands are required for optimal enzymatic activity in myocardial nuclei, but cortisone in large doses or aldosterone in near physiological doses is inhibitory. The identification of molecular mechanisms for these phenomena depends on detailed analytical and enzymological work, that is presently pursued. However, the organ-specific age and endocrine effects strongly support the view that poly(ADP-R) metabolism has direct relevance to differentiated organ functions in higher animals.

The organ-specific in vivo inhibitory effect of cortisol and especially of aldosterone that is demonstrable in normal hypophysectomized and adrenalectomized rats suggested a direct regulatory influence of steriods on nuclear poly(ADP-R) metabolism. If this is a direct effect, the inhibitory influence should be reproducible in vitro, which was indeed the case. Preincubation of isolated myocardial nuclei with 5.96 nM aldosterone for 30 minutes at 0° inhibits poly(APD-R) synthesis as treatment of rats in vivo with a daily dose of 5 µg aldosterone, indicating that a true organ-specific inhibitory effect of aldosterone is observed both in vivo and in vitro. These results are shown in Table III. It is unlikely that aldosterone actually acts directly as an inhibitor on the poly(ADP-R) polymerase enzyme. Our present working hypothesis predicts a specific interaction between aldosterone and a regulatory non-histone protein that participates

in the control of the poly(ADP-R) polymerase system. It is interesting that aldosterone given in vivo increases cardiac RNA-polymerase (Liew, Liu and Gornall, 1972). We presume that the poly(ADP-R) polymerase-non-histone protein modification system plays an intermediary role in the control of organ-specific transcription.

5. Summary: outline of regulatory sites and their probable function.

A deterministic role of specific non-histone proteins in the regulation of selective transcription of DNA segments by RNA polymerases is a plausible hypothesis that may explain the function of the large array of non-histone chromatin proteins in eukaryotes. The study of poly ADP-ribosylation affords a selective probe (Minaga et al., 1979) in this field. The modifying reagent NAD^+ is the only nucleotide which posesses rapid chemical responsiveness towards microenvironmental changes (i.e., oxidation/reduction). Stress induced reduction of NAD^+ will necessarily diminish protein ADP-ribosylation, as we have observed it in the hypertrophic part of the cardiac muscle that exhibits significant diminuation of poly(ADP-R) content as compared to the normal part of the same cardiac muscle (Minaga and Kun, unpublished experiments). This regulatory effect of decreased poly ADP-ribosylation that is presumably equivalent to a form of physiologic de-repression

coincides with an increased synthesis of all cellular macromolecules, (except DNA) resulting in hypertrophy. Much greater complexity exists when the effects of a carcinogen or of hormones are being followed. Because of the large number of non-histone chromatin proteins, each potentially regulating a discrete transcription of DNA-segments, no uniform relationship between the regulatory effects of individual non-histone proteins and their degree of ADP-ribosylation is likely. It is possible that activating effects of certain non-histone proteins (on transcription) may depend on ADP-ribosylation whereas with other proteins ADP-ribosylation is inhibitory. It follows that it is necessary to determine ADP-ribosylations of all non-histone proteins and establish an organ-specific pattern related to function before meaningful experimental questions can be formulated.

Our present information, regarding the role of ADP-ribosylations in sterol hormone action, suggests a possible connecting link between the sterol-specific nuclear receptors (Jensen, 1979) and the selective activation of RNA-polymerases. Our results with aldosterone in myocardial nuclei projects a feasible experimental approach.

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POLY ADP-R CONTENT, NAD^+ CONTENT AND ^{14}C-RIBOSE PULSE LABELING IN NORMAL AND PRECANCEROUS HAMSTER LIVER

No.	Treatment of hamsters	$(ADP\text{-}R)_n$ nmoles ADP-R/mg DNA	$(ADP\text{-}R)_n$ spec. act. μCi/nmole ADP-R	NAD^+ content nmoles/g liver	^{14}C-ribose spec. act. of NAD^+, μCi/nmole
1	Control (1 month)	22 ± 5	34 ± 8	646 ± 62	44 ± 8
2	DMNA treatment for 1 month	112 ± 38	58 ± 19	400 ± 40	83 ± 15
3	Control (2 months)	23 ± 5	2.9 ± 0.7	649 ± 60	5.3 ± 0.7
4	DMNA treatment for 2 months	38 ± 8	11.0 ± 2.6	550 ± 44	17.4 ± 3

LEGEND TO TABLE I

Eight male Syrian hamsters were selected on the basis of equal body weight within the chosen age groups. The hamsters (2 animals in each group) were deprived of food for 16 hours, then pulse labelled with 50 μCi ^{14}C-labelled ribose (58 mCi/mmole, ICN Pharmaceuticals) by intraperitoneal injection. Three hours after labelling liver homogenates were prepared in citric acid and nuclei were isolated. The total radioactive material per g liver homogenate, prepared from the control and the DMNA-treated hamsters, was identical. Quantitative isolation of $(ADP\text{-}R)_n$-protein adducts was carried out by boronate affinity chromatography. Analyses of $(ADP\text{-}R)_n$ were performed after either chemical or enzymatic hydrolysis of ADP-R-protein adducts, followed by HPLC-fluorometry of characteristic nucleotides. NAD^+ was determined in the post-nuclear supernatant after deproteinization with 5% $HClO_4$ and isolation by HPLC. The body weight of groups 1 and 2 were 80-100 g and of 3 and 4, 140-160 g. Analyses were done in triplicates and results show arithmetic mean values and standard deviation.

TABLE III

Inhibitory Effect of Aldosterone *In Vivo* and *In Vitro*
On the Poly(ADP-R) Synthetase Activity of Myocardial Nuclei

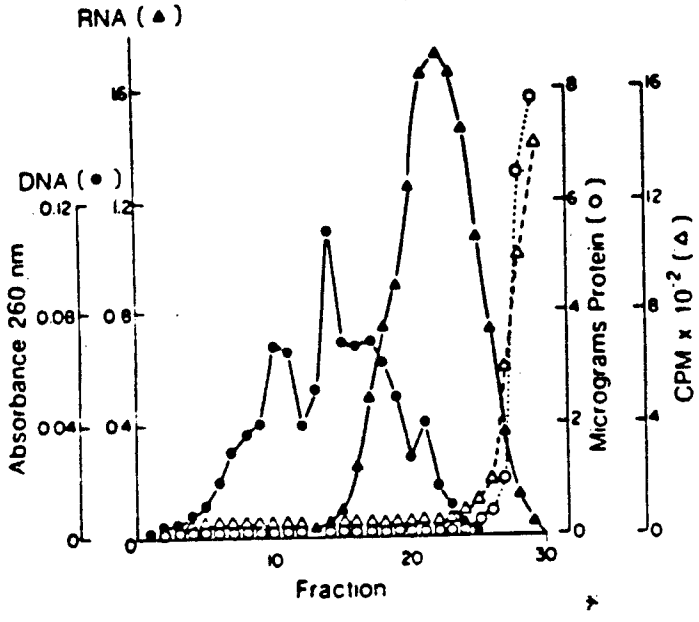
	Poly(ADP-R) Synthetase Activity (nmoles/mg protein/min)	% Inhibition
In Vivo		
control	8.8	
aldosterone (5µg/100 g rat)	5.6	36%
In Vitro		
control	5.6	
aldosterone (5.9 nM)	3.1	45%

LEGEND TO TABLE III

In vivo effect. Aldosterone was injected (5 µg/100 g body weight) intraperitoneally into 30-day-old Long Evans rats for four days, prior to the isolation of nuclei. Control received isotonic saline at the same time. Poly(ADP-R) synthetase activity was determined as described in Fig. 7. In all the Poly(ADP-R) synthetase assay the content of nuclear DNA per assay was the same.

In vitro effect of aldosterone on myocardial poly(ADP-R) synthetase activity. Myocardial nuclei from 30-day-old Long Evans male rats were preincubated at 0° for 30 min. in the presence or absence of 5.9 nM aldosterone. The system contained 100 mM Tris-HCl pH 8.2, 2 mM DTT, 20 mM $MgCl_2$, 0.1 mM PMSF, 0.5 mM EDTA. At the end of preincubation poly(ADP-R) synthesis was initiated by the addition of labelled NAD^+ (0.5 mM) and the synthesis carried out at 25° as described in legend of Fig. 7.

Fig 1.


RNA (▲)
DNA (●)
Absorbance 260 nm
0.12
0.08
0.04
0
1.2
0.8
0.4
0
16
8
4
0
12
8
4
0
Micrograms Protein (○)
CPM x 10⁻² (△)
10
20
30
Fraction

LEGEND TO FIG. 1

Separation of DNA (60 μg), RNA (400 μg) and ADP-R-protein adducts by ultracentrifugation in CsCl gradient. Time of centrifugation = 40 hours at 125,000 x g (av.) at 4°. (●——●) = 60 μg DNA, (▲——▲) = 400 μg RNA, (O– – –O) = ^{14}C-ribose $(ADP\text{-}R)_n$-protein counts, and (△– – –△) = protein content. DNA = thymus DNA; RNA = obtained from yeast.

Fig 2

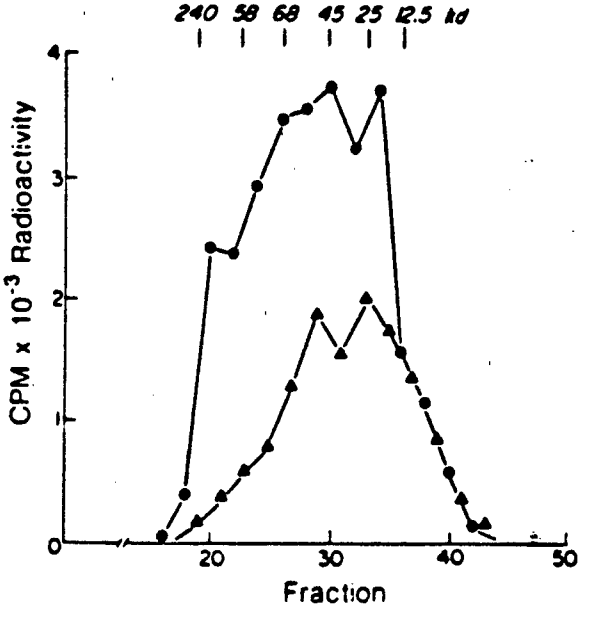
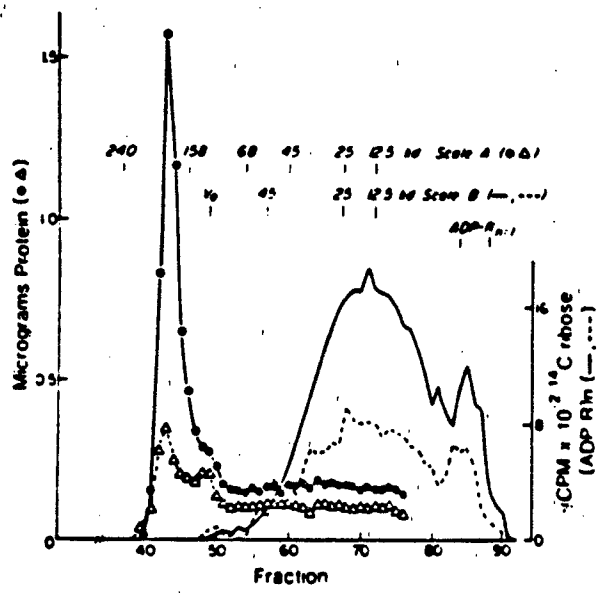

240 58 68 45 25 12.5 kd
CPM x 10⁻³ Radioactivity
0
1
2
3
4
20
30
40
50
Fraction

Fig 3

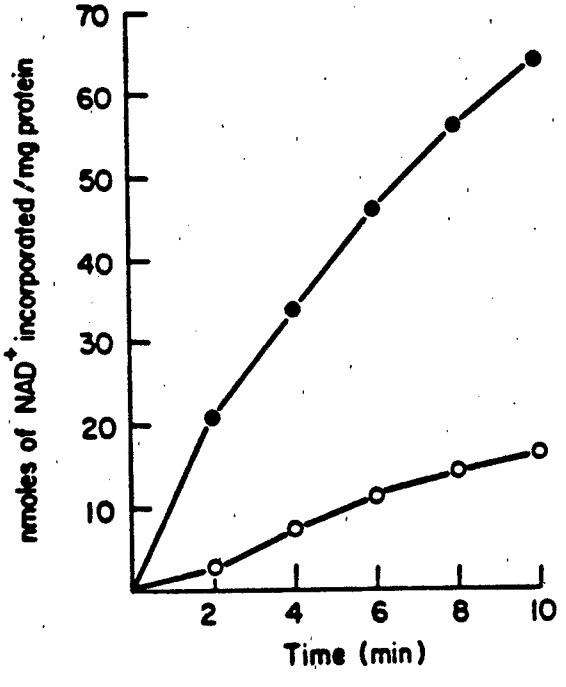

240
158
68
45
25
12.5 ml Scale A (o Δ)
V_0
45
25
12.5 ml Scale B (—, ---)
$ADP-R_{n-1}$
Micrograms Protein (o Δ)
CPM x 10^{-2} ^{14}C ribose (ADP Rib (—, ---))
Fraction
0
0.5
1.0
1.5
40
50
60
70
80
90
4
8

LEGEND TO FIG. 2

Molecular mass distribution of protein-$(ADP\text{-}R)_n$ adducts isolated from hamster nuclei (control and treated with DNMA* for 1 month) by the affinity chromatography method on a TSK-3000 SW HPLC molecular sieve. Developing solvent: 1.5 M guanidine-formate, pH 5.0, flow rate 0.4 ml/min.

▲——▲ = control, ●——● = dimethylnitrosamine treated.

Fig 6


70
60
50
40
30
20
10
nmoles of NAD+ incorporated / mg protein
2
4
6
8
10
Time (min)

LEGEND TO FIG. 6

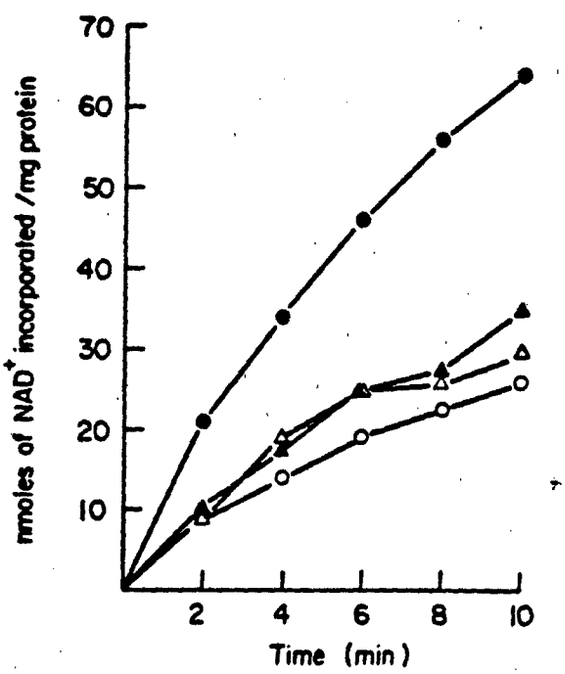
Changes in Myocardial Nuclear Poly(ADP-R) Synthetase Activity with Age

Myocardial nuclei isolated from 30-day-old and 65-day-old Long Evans rats were incubated with ^{14}C-NAD^+ and the incorporation of ^{14}C-NAD^+ into an acid insoluble product was monitored with time, as described in legend of Fig. 7.

● Myocardial nuclei (30 day old)

○ Myocardial nuclei (65 day old)

Fig 7


70
60
50
40
30
20
10
nmoles of NAD+ incorporated /mg protein
2
4
6
8
10
Time (min)

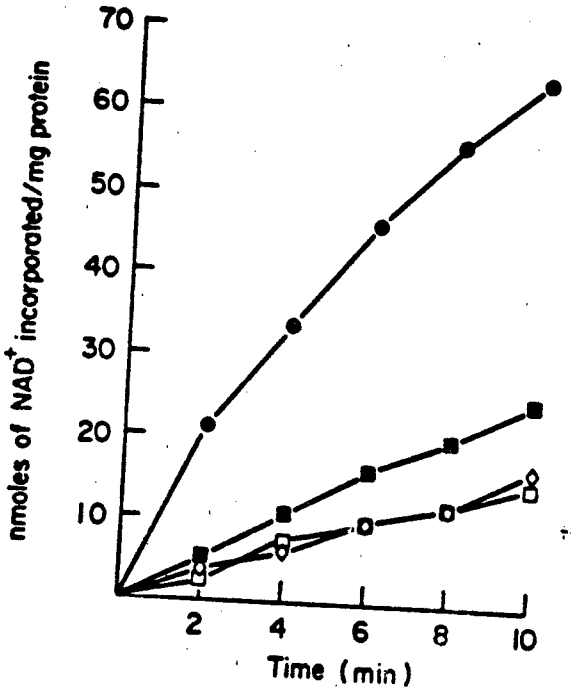
LEGEND TO FIG. 7

Poly(ADP-R) Synthetase Activity of Myocardial and Liver Nuclei From Cortisol-treated and Control Rats

Hydrocortisone sodium phosphate (Hydrocortone, MSD, 5 mg/rat) was injected intraperitoneally into 30-day-old Long Evans rats (100 g body weight) daily for 4 days, prior to the isolation of nuclei; a control group received isotonic saline at the same time. Poly-(ADP-R) synthetase activity in the isolated nuclei was monitored by measuring the rate of incorporation of ^{14}C-NAD$^+$ into an acid insoluble product. The reaction mixture, at 25° C in a volume of 235 µl contained 100 mM Tris HCl pH 8.2, 2 mM DTT, 20 mM $MgCl_2$, 0.1 mM PMSF, 0.5 mM EDTA, 0.5 mM NAD$^+$, 2.8 x 10^7 dpm of ^{14}C-NAD$^+$, 80 µg of nuclear material.

- ● Myocardial nuclei (control)
- ▲ Liver nuclei (control)
- △ Liver nuclei (cortisol-treated)
- ○ Myocardial nuclei (cortisol-treated)

~~53~~ Fig 8


nmoles of NAD+ incorporated/mg protein
70
60
50
40
30
20
10
2
4
6
8
10
Time (min)

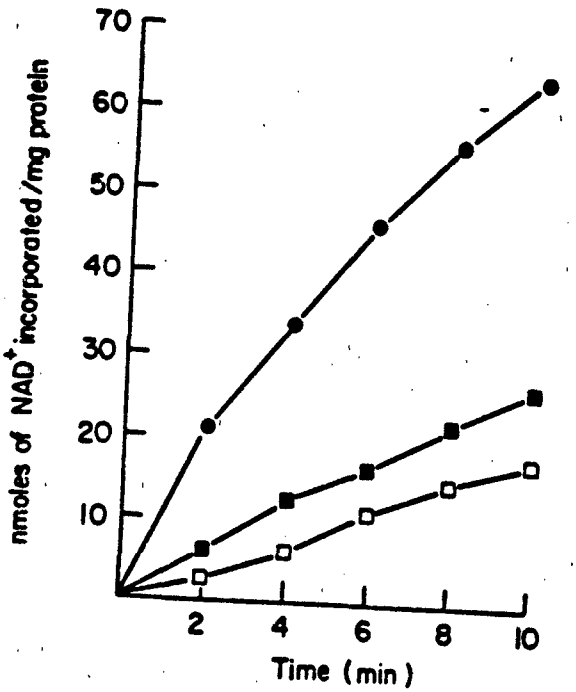
LEGEND TO FIG. 8

The Effect of Hypophysectomy, Cortisol and Aldosterone-treatment of Hypophysectomized Rats on Myocardial Nuclear Poly(ADP-R) Synthetase Activity

Hydrocortisone sodium phosphate (Hydrocortone, MSD; 5mg/100 g body weight) was injected intraperitoneally into hypophysectomized Long Evans rats daily for 4 days, prior to the isolation of nuclei. Aldosterone (Sigma; 5 µg/100 g body weight) was injected intraperitoneally into hypophysectomized rats following the same injection protocol employed for hydrocortisone; a control group consisting of normal rats and hypophysectomized rats received sterile isotonic saline. Poly(ADP-R) synthetase activity was monitored as described in Fig. 7.

- ● Myocardial nuclei (control)
- ■ Myocardial nuclei (hypophysectomized)
- ◇ Myocardial nuclei (hypophysectomized plus aldosterone)
- □ Myocardial nuclei (hypophysectomized plus cortisol)

Fig 9.


70
60
50
40
30
20
10
nmoles of NAD+ incorporated/mg protein
2
4
6
8
10
Time (min)

LEGEND TO FIG. 9

The Effect of Adrenalectomy and Cortisol-treatment of Adrenalectomized Rats on Myocardial Nuclear Poly(ADP-R) Synthetase Activity

Hydrocortisone sodium phosphate (Hydrocortone, MSD; 5 mg/100 g body weight) was injected intraperitoneally into adrenalectomized Long Evans rats daily for 4 days, prior to the isolation of nuclei; a control group consisting of normal rats and adrenalectomized rats received sterile isotonic saline. Poly(ADP-R) synthetase activity was monitored as described in Fig. 7.

- ● Myocardial nuclei (control)
- ■ Myocardial nuclei (adrenalectomized)
- □ Myocardial nuclei (adrenalectomized plus cortisol)